The manipulation of autophagy during early and late reperfusion: The effect on myocardial protection

by

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Declaration

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Marli Smit **5 October 2018**

Abstract

Introduction: Ischemic heart disease, the leading cause of death worldwide, often has devastating effects on myocardial function. Re-establishment of coronary flow to salvage myocardial cells results in reperfusion injury. There is an ongoing quest for effective therapeutic interventions against the deleterious effects of this phenomenon.

The pharmacological manipulation of autophagy, a process dealing with the organized destruction and recycling of cellular components, is one of the latest focus areas of research on cardioprotection against reperfusion injury. The significance of the autophagic response following ischemia/ reperfusion, as well as the effect of manipulation of this process on cardioprotection is still a matter of debate.

Aims: We hypothesised that autophagic induction during *early* reperfusion is protective while its upregulation during *late* reperfusion is detrimental. The *primary aim* was to investigate the protective effect of autophagic induction during early reperfusion and autophagic inhibition during late reperfusion. The secondary aim was to characterise autophagic flux patterns following different ischemic and reperfusion durations.

Methods: Isolated hearts from male Wistar rats were perfused in working mode. The control groups consisted of two ischemic (15 and 20 min) and 5 reperfusion (10, 30, 60, 90 and 120 min) intervals. In the interventional experiments 20min of global ischemia was followed by 30 (early) or 120 min (late) of reperfusion, during which 3 Methyl-adenine (3MA) and Rapamycin were used to inhibit and induce autophagy respectively. All experiments were repeated with Chloroquine, injection one hour prior to experimentation, to distinguish between steady state and autophagic flux. Western blotting was used to measure autophagic protein levels (LC3, Beclin, p62, DRP1, ULK1 and Rab9). Myocardial protection was measured assessing functional recovery and infarct size.

Results: The administration of Chloroquine, 3MA and Rapamycin, in the interventional groups, was without effect on global myocardial function before initiation of ischemia.

Western blotting: The *control experiments* demonstrated an increase in autophagic

steady state and flux during reperfusion, being more pronounced following longer ischemic and reperfusion durations. Early reperfusion administered 3MA caused a reduction in conventional and alternative autophagy during early reperfusion, as well as an increase in apoptotic activity. Rapamycin failed to induce autophagy during early reperfusion, but a high dose of Rapamycin resulted in an increase in autophagic flux during late reperfusion. High dose Rapamycin, during early and late reperfusion, additionally resulted in the inhibition of the alternative autophagic pathway.

Infarct size: The early reperfusion 3MA group demonstrated a significant decrease in infarct size when compared to all the other groups.

Conclusions: Our experimental model can be successfully used to study autophagy, and functional autophagy can be demonstrated up to (at least) 120 min reperfusion. Early reperfusion administered 3MA has cardioprotective properties, this may be attributed to the combination of the inhibition of the conventional and alternative autophagic pathways, apoptotic induction and direct drug effects. Late reperfusion induced autophagy was without effect on cardioprotection. We were unable to convincingly induce autophagy during early reperfusion and inhibit autophagy during late reperfusion, this is mainly attributed to experimental model reperfusion duration restrictions and unexpected difficulties experienced with pharmacological manipulation of autophagy in the model used.

Opsomming

Inleiding: Isgemiese hartsiekte is die hoofoorsaak van wêreldwye sterftes en het ‘n nadelige uitwerking op miokardiale funksie. Die herstel van koronêre bloedvloei, in ‘n poging om die miokardiale selle te red, lei tot herperfusie besering. Die soeke na suksesvolle behandelings opsies teen herperfusie besering is dus van kardinale belang.

Die farmakologiese manipulasie van outofagie, ‘n proses wat die georganiseerde afbraak en herwinning van sellulêre komponente behels, is een van die nuutste navorsingsareas wat op kardiale beskerming tydens herperfusie besering fokus. Die belang van outofagie se respons op isgemie en herperfusie, asook die effek van manipulasie daarvan op kardiale beskerming, word steeds gedebatteer.

Doelwitte: Ons hipotese is dat induksie van outofagie tydens vroeë herperfusie beskermend, en opregulering van die proses tydens laat herperfusie skadelik is. Die hoofdoel was dus om die beskerming van die hart deur outofagiese induksie tydens vroeë herperfusie en inhibisie tydens laat herperfusie te ondersoek. ‘n Verdere doelwit was om die patroon van outofagiese fluks, wat op verskillende isgemiese en herperfusie periodes volg, te beskryf.

Metodes: Geisoleerde harte van manlike Wistar rotte, is geperfuseer volgens die werkhart tegniek. Die kontrole groepe het bestaan uit twee isgemiese (15 en 20 min) en vyf (10, 30, 60, 90 and 120 min) herperfusie intervalle. Tydens die intervensionele eksperimente is 20 min globale isgemie gevolg deur 30 (vroeë) of 120 (laat) min herperfusie. 3 Metiel-adenine (3MA) en Rapamycin is toegedien tydens herperfusie om onderskeidelik outofagie te inhibeer en te induseer. Alle eksperimente is herhaal met een uur pre-eksperimentele Chloroquine toediening om te onderskei tussen bestendige staat outofagie en outofagiese fluks. Outofagiese proteïenuitdrukking (LC3, Beclin, p62, DRP1, ULK1 en Rab9) aktiwiteit is met behulp van die Western-klad tegniek ge-analiseer. Hemodinamiese en infarktgrootte bepalings is gebruik om die mate van miokardiale beskerming te bepaal.

Resultate: Chloroquine, 3MA en Rapamycin toediening, tydens die intervensionele eksprimente, het nie globale miokardiale funksie voor die aanvang van isgemie beïnvloed nie.

Western-klad: Die kontroles het ‘n toename in outofagiese bestendige staat en fluks tydens herperfusie getoon wat meer uigesproke was met langer isgemiese en herperfusie periodes. 3MA toediening tydens vroeë herperfusie het inhibisie van die tradisionele asook die alternatiewe outofagiese pad, sowel as ‘n toename in apoptose, veroorsaak. Hoë dosering Rapamycin, toegedien tydens laat herperfusie, het ‘n toename in outofagiese flux meegebring, sowel as ‘n inhibisie van die alternatiewe pad tydens beide vroeë en laat herperfusie.

Infarkgroottes: Vroeë reperfusie toegediende 3MA het, in vergelyking met al die ander groepe, ‘n beduidende afname in infarkgrootte gedemonstreer.

Gevolgtrekkings: Ons eksperimentele model kan suksesvol aangewend word om outofagie te bestudeer, en funksionele outofagie is gedemonstreer tot en met 120 min herperfusie. 3MA toediening tydens vroeë herperfusie is kardiobeskermend wat toegeskryf kan word aan ‘n kombinasie van die inhibisie van die tradisionele en alternatiewe autofagiese paaie, apoptotiese induksie en direkte middel-afhanklike aksies. Laat herperfusie outofagiese induksie het geen nadelige effekte op die hart gehad nie. Outofagiese induksie tydens vroeë reperfusie en inhibisie tydens laat reperfusie was onsuksesvol. Dit word toegeskryf aan beide die beperkte herperfusie periode van die eksperimentele model asook onverwagte probleme ondervind met die farmakologiese manipulasie van outofagie in die betrokke model.

Dedication

This thesis is dedicated to my Dad, Deon Smit.

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List of acronyms and abbreviations

3MA	3 Methyl-adenine
A	Ampere
AB	Antibody
ACE	Angiotensin converting enzyme
AMI	Acute myocardial infarction
AMPK	5'-AMP activated protein kinase
AMPK	Adenosine monophosphate-activated protein kinase
AngII	Angiotensin II
ANOVA	One-way analysis of variance
Atg	Autophagy related genes
AU	Arbitrary units
Bcl-2	B-cell lymphoma/leukemia-2
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
Bpm	Beats per minute
CAD	Coronary artery disease
CaO2	Arterial oxygen content
CBF	Coronary blood flow
CCD	Central coiled domain
CK-MB	Creatine kinase muscle/brain
CMA	Chaperone-mediated autophagy
CO	Cardiac output
CPP	Coronary perfusion pressure
CQ	Chloroquine
DISC	Death Inducing Signaling Complex
DO2	Oxygen delivery
DRP1	Dynamin- related protein 1
Duox	Dual oxidases

ECD	Evolutionarily conserved domain
ECG	Electrocardiograph
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ETC	Electron transport chain
FADD	Fas-associated protein with death domain
FIP200	Focal adhesion kinase family-interacting protein of 200 kD
FMHS	Faculty of Medicine and Health Sciences
GERL	Golgi endoplasmic reticulum lysosomes
GI	Global ischemia
GIK	Glucose-insulin-potassium
GSK3	Glycogen synthase kinase-3 beta
Hb	Haemoglobin
$HCO3^-$	Bicarbonate
HIF	Hypoxia inducible factor
HR	Heart rate
I/R	Ischemia/reperfusion
ICAM-1	Intercellular Adhesion Molecule 1
IHD	Ischemic heart disease
IL-1	Interleukin-1
IP	Intraperitoneal
IP3	Inositol-1,4,5-triphosphate
KHB	Krebs-Henseleit buffer
L	Langendorff mode
LADCA	Left anterior descending coronary artery
LAMP	Lysosomal-associated membrane protein
LAP	LC3-associated phagocytosis
LAD	Left anterior descending

LC3	Light chain 3
LDL	Low density lipoprotein
LT	Leukotriene
LVEDP	Left ventricular end diastolic pressure
M-CSF	Macrophage colony stimulating factor
MCP-1	Monocyte chemoattractant protein 1
Min	Minutes
mPTP	Mitochondrial Permeability Transition Pore
mTORC1	Mammalian target of Rapamycin complex 1
MVO	Microvascular obstruction
MW	Molecular weight
NADPH	Nicotinamine adenine dinucleotide phosphate oxidase,
NCCD	Nomenclature Committee on Cell Death
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	NO synthase
Nox4	NADPH oxidase 4
NSTEMI	Non-ST segment elevation infarction
O_2	Oxygen
$ONOO^-$	Peroxynitrite
OXPHOS	Oxidative phosphorylation
p62/SQSTM1	Sequestosome 1
PAF	Platelet-activating factor
PAO	Aortic pressure
PaO_2	Partial pressure of oxygen
PCD	Programmed cell death
PE	Phosphatidylethanolamine
PGI2	Prostacyclin

PI	Post ischemic
PIKC3	Phosphatidylinositol kinase complex class three
PLA	Phospholipase
PMN	Polymorph nuclear
PPCI	Primary percutaneous coronary intervention
Pre-I	Pre-ischemic interval
PSP	Peak systolic pressure
PVDF	Polyvinylidene fluoride membranes
Q	Flow
Qa	Aortic flow
Qc	Coronary flow
R	Reperfusion
R-PCI	Rescue percutaneous coronary intervention
RAA	Renin–angiotensin–aldosterone
Rab	Ras related protein
RI	Regional ischemia
RIC	Remote ischemic conditioning
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rpm	Rounds per minute
SANS	South African National Standards
SaO_2	Oxygen saturation
SAVC	South African Veterinary Council
SEM	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum Ca2+-ATPase
SKD	Suppressor of potassium transport growth defect
SMERs	Small-molecule enhancers of the cytostatic effects of rapamycin
SNARE	Soluble N-ethylmaleimide–sensitive factor attachment protein receptor

SOD	Superoxide dismutase
STEMI	ST segment elevation infarction
SU	Stellenbosch University
TBST	Tris-buffered saline-Tween-20
TCA	Tricarboxylic acid
TFEB	Transcription factor EB
TNF-α	Tumor necrosis factor alpha
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TTC	Triphenyltetrazolium chloride
ULK	Unc-51 like autophagy activating kinase
UPS	Ubiquitin proteasome system
UVRAG	UV radiation resistance associated gene
V	Volt
VCAM-1	Vascular cell adhesion molecule 1
VO_2	Oxygen consumption
Vps	Vacuolar protein sorting
WH	Working heart
Wt	Total work
Wtot	Total work
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
Zo	Tissue pressure
η	Viscosity

List of units of measurement

%	Percentage
°C	Degree Celsius
AU	Arbitrary units
bpm	Beats per minute
cm	Centimeter
dL	Deciliter
g	Gram
IU	International units
kDa	Kilo Dalton
kg	Kilogram
kJ	Kilojoules
L	Liter
m	Meter
M	Molar
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
mmHg	Millimeters of mercury
mmol	Millimol
mW	Milliwatt
N	Normality
ng	Nanogram
nM	Nanomolar
rpm	Revolutions per minute
v	Volume

µ	Micro
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar
µmol	Micromol

Literature review: Introduction

Myocardial ischemia occurs when blood flow to the myocardium is diminished secondary to a partial or complete blockage of a coronary artery. Myocardial infarction can and often has devastating effects on myocardial function, (de Zwaan, Daemen and Hermens, 2001) and is the principle contributor to morbidity and mortality in the world (Mozaffarian *et al.*, 2015). The only way to lessen the detrimental effects associated with ischemia is to re-establish the flow to the myocardial cells, the process of reperfusion.

Unfortunately, reperfusion following ischemia, although essential for survival, also adds more insult by causing the death of cells that were still viable at the end of ischemia. This phenomenon is referred to as reperfusion injury (RI), and is responsible for up to half of the final infarct size (Hausenloy and Yellon, 2013). Currently there is no effective therapeutic intervention to entirely prevent the deleterious effects of reperfusion injury (Hausenloy and Yellon, 2013; Thapalia, Zhou and Lin, 2014).

Autophagy (from the Greek *auto*-, "self" and *phagein*, "to eat") is a normal physiological process in the body that deals with the organized destruction and recycling of cellular components. It contributes to homeostasis through the turnover of destroyed cell organelles for new cell formation (Kobayashi, 2015).

The manipulation of autophagy is one of the latest areas of research in the field of myocardial protection against ischemia and reperfusion. The majority of findings show that autophagy is upregulated during ischemia and reperfusion (Gustafsson and Gottlieb, 2009). Whether upregulated autophagy is beneficial or detrimental in terms of myocardial protection is however still a matter of debate. We propose that this will depend on the degree and timing (in relation to reperfusion) of autophagic upregulation.

Myocardial protection secondary to the pharmacological manipulation of autophagy during early and late reperfusion has not been studied previously. **The aim of this study is to explore and investigate whether manipulation of autophagy reduces reperfusion injury**. In view of this, the literature review will provide a summary of our

current knowledge regarding autophagy, myocardial ischemia/reperfusion as well as the physiological role of autophagy in ischemia/reperfusion.

CHAPTER 1
AUTOPHAGY

1.1. INTRODUCTION

The process of 'self-eating' (autophagy in Greek) was initially observed in 1962 by Ashford and Porter in rat liver cells (Ashford and Porter, 1962). This observation consisted of the degradation of mitochondria and other intra-cellular structures within lysosomes of rat liver perfused with glucagon. Thereafter the term 'autophagy' was coined by the Belgian biochemist Christian de Duve in 1963 during the *Ciba Foundation Symposium on Lysosomes,* which took place in London (Klionsky, 2008; Thapalia, Zhou and Lin, 2014).

The term autophagy is therefore used to allude to the degradation of cytoplasmic components within lysosomes (Levine and Klionsky, 2004; Mizushima, 2007).

Recently the scientific world 'rediscovered' autophagy, realizing the physiological significance of this process (Deter and De Duve, 1967; Barth, Glick and Macleod, 2010). Studies clearly demonstrated that autophagy has a great variety of physiological and pathophysiological roles; this includes starvation adaptation, intracellular protein and organelle clearance, development, anti-aging, elimination of microorganisms, cell death, tumor suppression and antigen presentation (Mizushima, 2005, 2007). The importance of autophagy is well recognized in mammalian systems, however, most of the breakthroughs in the mechanistic aspects of autophagy have been made in yeast (*Saccharomyces cerevisiae* and the methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha*) (He and Klionsky, 2009; Ylä-Anttila, Vihinen, Jokitalo and E.-L. Eskelinen, 2009; Glick, Barth and Macleod, 2010).

1.2. DIFFERENT TYPES OF AUTOPHAGY

Three different types of autophagy exist:

- Macro-autophagy,
- Micro-autophagy and
- Chaperone-mediated autophagy (CMA) (Mizushima *et al.*, 2008).

During macro-autophagy cytoplasmic components are engulfed by the autophagosome, (Gustafsson and Gottlieb, 2009) which then fuses with the lysosome to form an autophagolysosome. In microautophagy cytosolic components are directly taken up by the lysosome itself through invagination of the lysosomal membrane. In CMA, targeted proteins are translocated across the lysosomal membrane with the help of chaperone proteins that are recognized by the lysosomal membrane receptor, lysosomal-associated membrane protein 2A (LAMP-2A), which results in their degradation (Eskelinen, 2008b; Glick, Barth and Macleod, 2010).

Thus, in macroautophagy and chaperone–mediated autophagy the cytoplasmic components need to be transported to the lysosomes for degradation. Microautophagy on the other hand, requires no transportation (Thapalia, Zhou and Lin, 2014) since uptake occurs directly at the lysosomal membrane.

Of the three types mentioned, macroautophagy is the most well studied process. In this thesis, macro-autophagy will be implied when the term autophagy is used.

1.3. THE AUTOPHAGIC PROCESS

Autophagy (in all three forms) is responsible for the degradation of long-lived proteins, RNA, macromolecules and excess or dysfunctional organelles. This is achieved by means of lysosome consumption (Cuervo, 2004; Levine and Klionsky, 2004) and is also known as *type II programmed cell death*.

In short: autophagy starts with the formation of an isolation membrane (also known as a phagophore). This phagophore expands to engulf intracellular cargo (such as protein aggregates, organelles, invasive pathogenes and ribosomes) in a double-membrane autophagosome (Klionsky *et al.*, 2008). The loaded autophagosome then transports the cargo to the lysosomes where the outer-membrane of the autophagosome fuses with the lysosomal membrane (creating an autophagolysosome), after which the inner vesicle, together with its cargo, is degraded by lysosomal acid proteases (He and Klionsky, 2009). The resulting products of degradation, can be recycled back to the cytosol for reuse during starvation, (Yorimitsu and Klionsky, 2005) for building macromolecules and for metabolism (Klionsky *et al.*, 2008; He and Klionsky, 2009; Glick, Barth and Macleod, 2010).

Autophagy may therefore be viewed as a 'recycling factory', promoting energy efficiency and facilitating damage control by removing non-functional proteins and organelles (Glick, Barth and Macleod, 2010). This efficient sequestration and clearance of surplus or damaged cellular or non-self cellular components, are important for normal cell function and survival (He and Klionsky, 2009).

A dysregulated autophagic response however also exists. This may contribute to many disease processes and will be discussed in more detail in Chapter 4.

1.4. THE MECHANISTIC ASPECTS OF AUTOPHAGY

The process of autophagy can be divided into distinct mechanistic phases (Gatica *et al.*, 2015):

a. Trigger and induction.
b. Autophagosome formation; consisting of nucleation, elongation and maturation.
c. Cargo recognition.
d. Autophagsome-lysosome fusion.
e. Breakdown of the cargo followed by release of the degradation products back into the cytosol.

Autophagosomes have been observed by electron microscopy as early as the 1950s, (Klionsky, 2007) but the molecular era of autophagy began only a little more than a decade ago. This originated mainly from genetic screening in yeast, which lead to the identification of 31 autophagy-related genes (Atg). Different gene protein sets are involved and responsible for the different phases and steps of autophagy (He and Klionsky, 2009). Autophagy can accordingly be divided into the above mentioned phases based on the proteins involved (Gatica *et al.*, 2015).

a. Trigger and induction*:*

Although autophagy occurs continuously at basal level, it can be induced in many cells. Since autophagic activity is low under normal conditions, an efficient mechanism to induce autophagy is required for organisms to adapt to stressors and changes in their nutrient status. Low energy conditions (hypoglycaemia/nutrient starvation and hypoxia) are the most powerful stimuli for autophagy; the reverse environment inhibits autophagy. Particularly in the heart, autophagy is upregulated by starvation, reactive

oxygen species (ROS), hypoxia, exercise and ischemic preconditioning (refer to Section 3.5.1, Ischemic conditioning) (Gottlieb, Finley and Mentzer, 2009; Thapalia, Zhou and Lin, 2014).

In cardiomyocytes, glucose deprivation and ischemia result in activation of NADPH oxidase 4 (Nox4), a ROS-producing enzyme. The increase in ROS promote autophagy by activating the PERK/ATF4 pathway (Sciarretta *et al.*, 2013).

Growth factors, hormones, receptors with tyrosine kinase activities, Bcl-2, calcium (Høyer-Hansen *et al.*, 2007) and myo-inositol-1,4,5-triphosphate (IP_3) (Sarkar *et al.*, 2005; Mizushima, 2007) can also regulate autophagy (Thapalia, Zhou and Lin, 2014). For a summary of autophagic triggers, see Table 1.1.

Table 1.1: Physiological and pharmacological triggers involved in autophagic regulation

Induction of autophagy	**Inhibition of autophagy**
Low energy conditions	High energy conditions
Hypoglycemia/ nutrient starvation	Growth conditions/ nutrient rich environment
Rapamycin	3 Methyl-adenine
Glucagon	Insulin
Exercise	Growth factors
Calcium	AMPK (5'-AMP activated protein kinase) (Fig 1.3)
Reactive oxygen species	Wortmannin
Ischemic preconditioning	
Hypoxia	
Hyperoxia	

This induction and regulation of autophagy on a molecular/mechanistic level appears to be quite complicated. For instance, total amino acid depletion strongly induces autophagy, while the effects of individual amino acids are different. How cells sense amino acid concentration is not fully understood (Nobukuni *et al.*, 2005). It appears as if hormones, growth factors and the endocrine system (with the emphasis on insulin) manage autophagy regulation *in vivo* (Mizushima, 2007).

Phosphatidylinositol kinase complex class three (PIKC3) is a human core complex involved in the signaling pathways for the induction of autophagy. This complex consists of three major components: (Refer to Figure 1.1)

- PIKC3 /Vps34 (Phosphatidylinositol kinase complex class three, which translates to vacuolar protein sorting 34 in yeasts)
- p150 /Vps15 and
- Beclin 1/Vps30 (also known as Atg6) (Sun, Fan and Zhong, 2009).

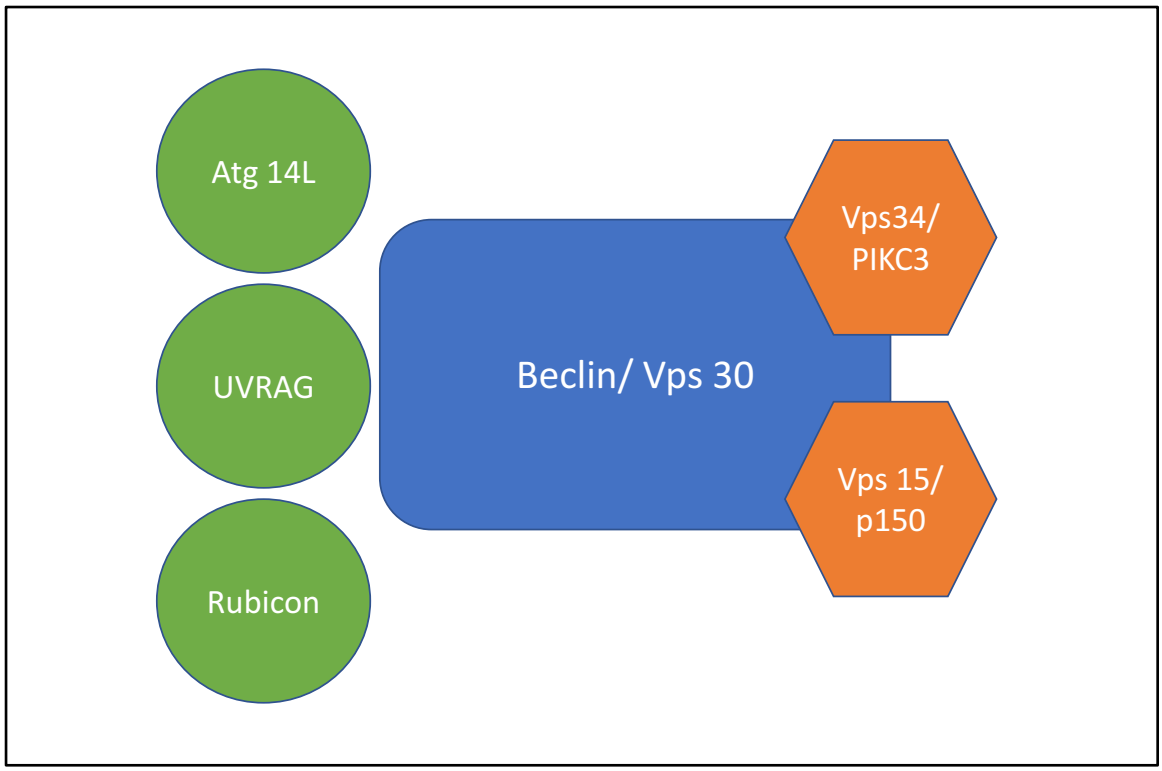


Figure 1.1: Phospatidylinositol kinase complex class three and associated protein complexes

This complex consists of three components, PIKC3 /Vps34, p150 /Vps15 and Beclin. The three different protein options to associate with Beclin include Atg14L, UVRAG and Rubicon. Abbreviations: PIKC3: Phosphatidylinositol kinase complex class three, VPS: vacuolar protein sorting, Atg: autophagy related gene and UVRAG: UV radiation resistance associated gene.

Three variations of above-mentioned Beclin 1-Vps34-Vps15 complexes exist which depend on the Beclin associated protein connected to the complex. The options are (Figure 1.1):

1. Atg14L,
2. UVRAG (UV radiation resistance associated gene) or

3. Rubicon (Kang *et al.*, 2011).

Atg14L is localized on the isolation membrane of the autophagosome and plays a part in phagopore formation and induction. Rubicon takes part in the inhibition of autophagy, especially during the maturation step (Matsunaga *et al.*, 2009). The UVRAG complex is required for autophagosome maturation and lysosome fusion (Sun, Fan and Zhong, 2009). The phosphatidylinositol kinase complex can therefore fulfill different functions, depending on the subunit composition (Matsunaga *et al.*, 2009).

Beclin 1 has three domains (Figure 1.2): The BH3 domain, central coiled domain (CCD) and the evolutionarily conserved domain (ECD). Bcl-2 interacts with the BH3 domain, UVRAG with the CCD, and the class III PIKC3-kinase with the ECD as well as the CCD (Matsunaga *et al.*, 2009; Abounit, Scarabelli and McCauley, 2012).

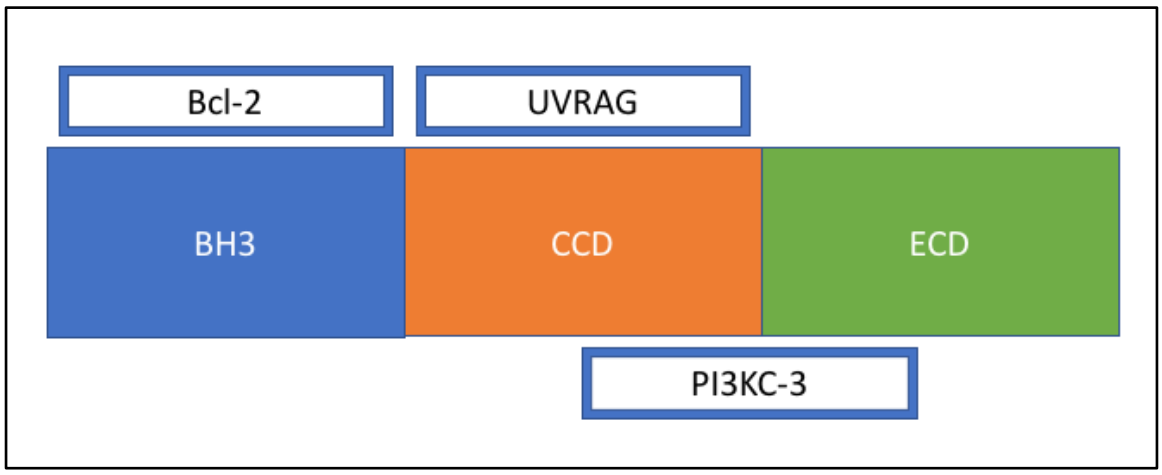


Figure 1.2: Beclin-1 domains

Abbreviations: CCD: central coiled domain, ECD: evolutionarily conserved domain, UVRAG: UV radiation resistance associated gene, PIKC3: Phosphatidylinositol kinase complex class three, Bcl-2: B-cell lymphoma/leukemia-2. (Drawing modified from Liang *et al.* (2006))

The mammalian target of Rapamycin complex 1 (mTORC1) – also known as the master regulator of nutrient signaling – is the most important focus point of the autophagic triggers. Under resting conditions, phosphorylated (active) mTORC1 is bound to and phosphorylates Unc-51- like kinase 1 (Ulk1), (Figure 1.3). During starvation AMP accumulates as ATP is depleted. This increase in AMP activates AMPK (5'-AMP activated protein kinase), which is responsible for TSC1/2 activation, which will inhibit Rheb, an mTORC1 activator (Inoki *et al.*, 2006). Summarizing above

– AMPK activation will result in the dephosphorylation (inhibition) of mTOR, (Kim *et al.*, 2011) and therefore activation of autophagy (Sciarretta *et al.*, 2018). AMPK may also directly activate autophagy by phosphorylating Ulk1 from mTORC1 (Kim *et al.*, 2011). Glycogen synthase kinase-3 beta (GSK3) is another important regulator of autophagy. It is being activated during ischemia resulting in suppression of mTORC1 activity. GSK3 is inhibited during reperfusion, which will allow mTORC1 activation and limiting autophagy (Zhai *et al.*, 2011; Sciarretta *et al.*, 2018). Energy rich conditions are also responsible for mTORC1 activation and therefore inhibition of autophagy. Not all autophagic signals are transduced through mTOR. Small-molecule enhancers of the cytostatic effects of rapamycin (called SMERs), may also induce autophagy, and act independently of mTOR (Mizushima, 2007; Sarkar *et al.*, 2007).

In yeast, Atg 1 is activated upon TOR inhibition; this leads to the formation of an Atg1-Atg13-Atg17 complex, which initiates autophagosome formation (Kabeya *et al.*, 2005). The role of the Atg1 kinase complex in autophagic induction is therefore very important in *yeasts*. There are two mammalian homologs of Atg1, the Unc-51- like kinase 1 (ULK1) and -2 (ULK2). There is one homolog of yeast Atg17, known as FIP200 (the focal adhesion kinase family-interacting protein of 200 kD). FIP200 forms a complex with ULKs and mammalian Atg13 and then localizes to the phagophore upon starvation (Hara *et al.*, 2008; Jung *et al.*, 2009). All of above is conducive in terms of autophagy **induction**. During nutrient rich conditions mTOR phosphorylates and inactivates ULKs and Atg13. During nutrient depleted conditions the mTOR inhibition will activate ULK1 and ULK2 and phosphorylate Atg13 and FIP200 (Hosokawa *et al.*, 2009; Jung *et al.*, 2009). The activated ULK complex then becomes associated with the phagophore and **initiates formation of the autophagosome** (Ganley *et al.*, 2009). Atg101 binds and stabilizes Atg13, and is therefore also required for autophagy in mammals (He and Klionsky, 2009; Mercer, Kaliappan and Dennis, 2009). This activated ULK1 complex phosphorylates Beclin-1, enhancing the activity of the previously mentioned Atg14L-containing Beclin 1-Vps34-Vps15 (PIKC3) complexes. The Beclin-1 phosphorylation by ULK is therefore required for full autophagic induction in mammals (Russell *et al.*, 2013).

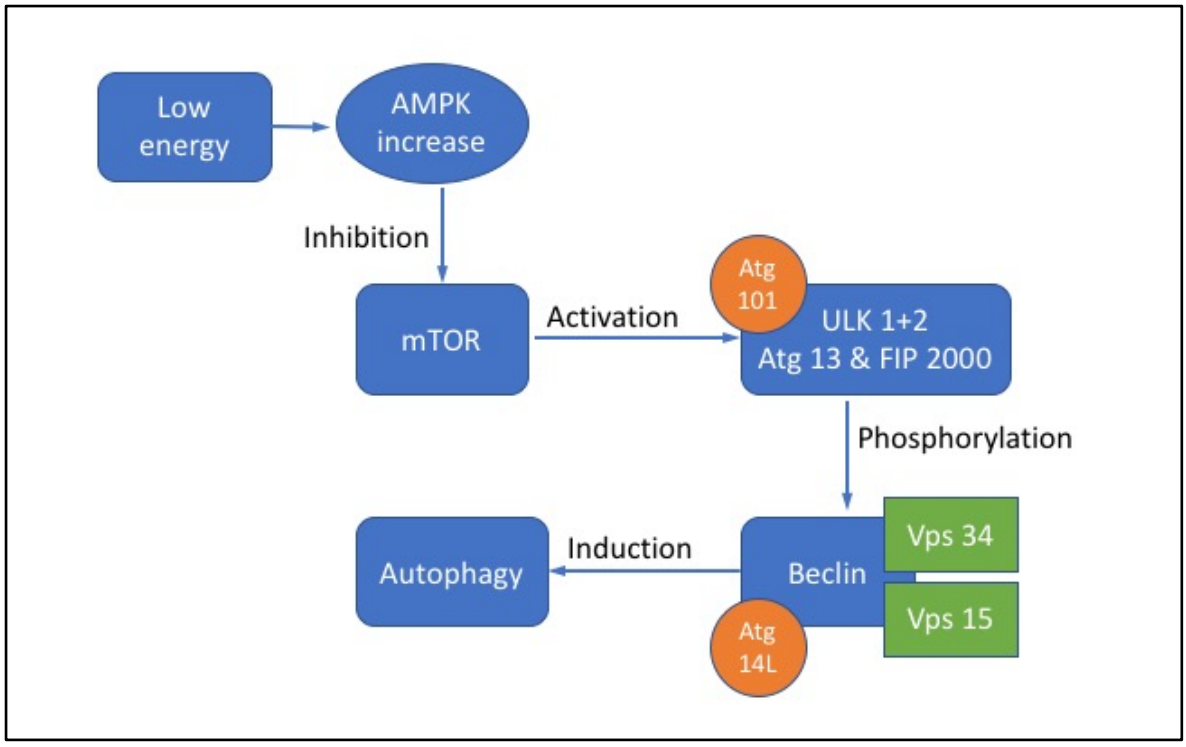


Figure 1.3: Schematic representation of autophagic induction

Abbreviations: FIP200: the focal adhesion kinase family-interacting protein of 200 kD, ULK: Unc-51-like kinase, Atg: autophagy related gene, VPS: vacuolar protein sorting, mTOR: mammalian target of Rapamycin, AMPK: 5'-AMP activated protein kinase.

The best studied autophagy related gene, Beclin 1/Atg6, could be responsible for another mechanism by which nutrient starvation induces autophagy. Beclin 1, localized at the core of autophagy regulation (Cao and Klionsky, 2007), was originally identified as an interactive partner of Bcl-2 (B-cell lymphoma/leukemia-2), an anti-apoptotic protein. This (Bcl-2–Beclin 1) interaction is reduced upon starvation, liberating Beclin 1 to activate autophagy (Figure 1.4) (Pattingre *et al.*, 2005; M. C. Maiuri *et al.*, 2007).

Bcl-2 inhibits autophagy by competing with Vps34 for Beclin-1 binding (Abounit, Scarabelli and McCauley, 2012).

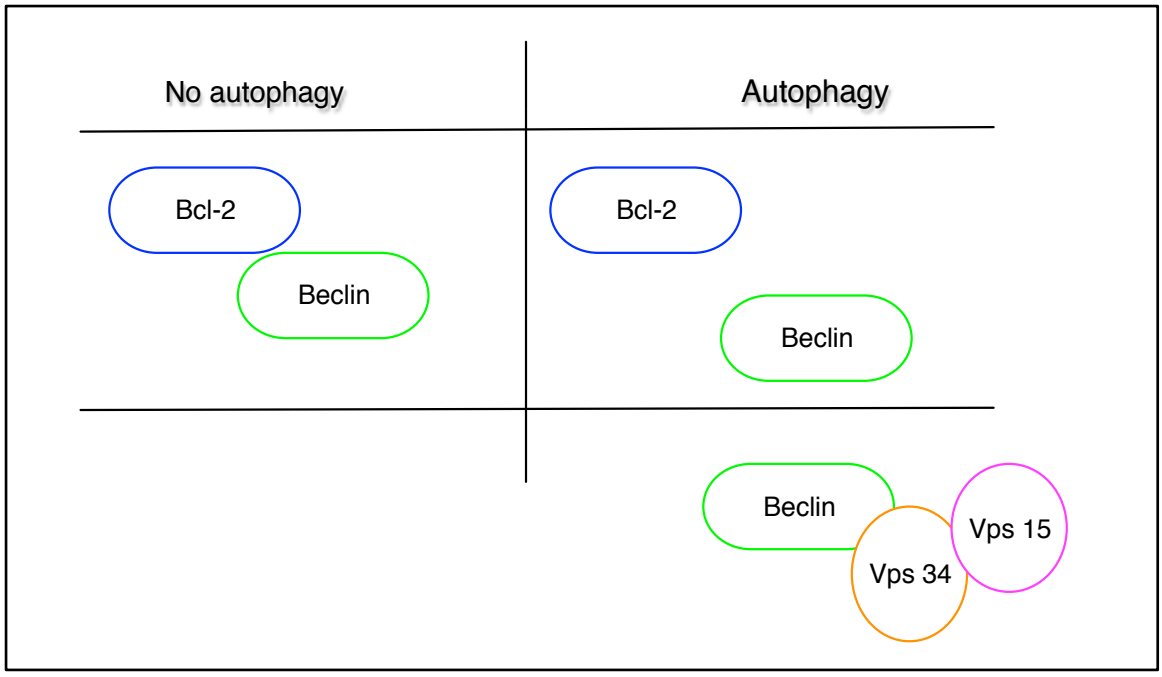


Figure 1.4: Schematic representation of the Bcl-2–Beclin interaction in the induction of autophagy

Upon starvation, the Bcl-2-Beclin interaction is reduced, freeing Beclin to activate autophagy. Abbreviations: Bcl-2 (B-cell lymphoma/leukemia-2) VPS: vacuolar protein sorting.

On the other hand, phosphorylation of Beclin 1 by Mst1 (a serine/threonine kinase) enhances its interaction with Bcl-2. This interaction will inhibit autophagy, promoting the dissociation of Bcl-2 from Bax, thereby stimulating apoptosis. Pro-apoptotic stress-induced activation of Mst1 promotes cell death through both autophagic suppression and stimulation of apoptosis (Maejima *et al.*, 2013).

b. Autophagosome formation; nucleation, elongation and maturation: (Refer to Figure 1.5 and 1.6)

Any of the above-mentioned triggers would eventually lead to induction of autophagy and autophagosome formation. This process starts with formation of a **phagophore** (isolation membrane), a cell membrane protrusion that acts as a precursor membrane to mammalian autophagosomes (Axe *et al.*, 2008; Hayashi-Nishino *et al.*, 2009). The membrane source for the autophagosome is still a matter of uncertainty. There is evidence supporting the Golgi complex, the endoplasmic reticulum (ER), the mitochondria and an area of the cell termed GERL (Golgi endoplasmic reticulum lysosomes). In mammalian cells, however, phagophore membranes appear to initiate primarily from the endoplasmic reticulum (Hayashi-Nishino *et al.*, 2009; Glick, Barth

and Macleod, 2010). This pre-autophagosomal double membrane cup-shaped structure will surround a portion of the cytoplasmic content. It will then close off, engulfing the cytoplasmic content to create a double-membrane vacuole termed an autophagosome (Gustafsson and Gottlieb, 2008b; Iwai-Kanai *et al.*, 2008). As stated previously, the cytoplasmic content for sequestration includes invading pathogens, proteins, lipids and even damaged or surplus organelles (He and Klionsky, 2009; Gatica *et al.*, 2015).

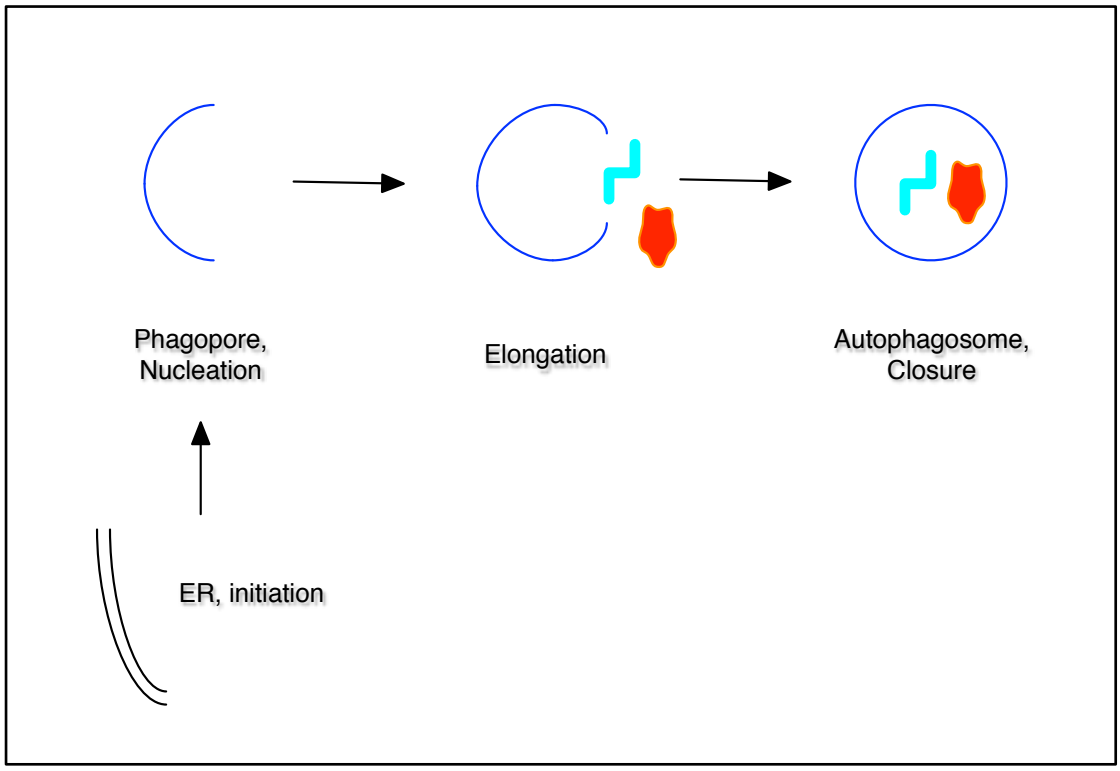


Figure 1.5: Autophagosome formation

The phagopore originates from the ER, the extension and the elongation of the membrane results in a cup shaped isolation membrane than can engulf cytoplasmic organelles (red and blue figures) and close to form the autophagosome. Abbreviation: ER: endoplasmic reticulum.

As discussed earlier, the activation of appropriate signaling pathways will lead to the initial step in the maturation of the autophagosome, namely inactivation of mTOR and its dissociation from a complex analogous to the yeast Atg1 complex. Subsequently, the PIKC class three assembles, resulting in the recruitment of multiple autophagy related gene proteins (Atg 18, 20, 21 and 24) that associates with the phagophore membrane. (Ravikumar *et al.*, 2010) This leads to the conjugation of two ubiquitin-like protein systems responsible for the **elongation** and extension of the phagophore (Abounit, Scarabelli and McCauley, 2012).

These protein systems are: (Figure 1.6)

- Atg12-Atg5 which consequently oligomerizes with Atg16 and
- Microtubule associated protein light chain 3-phosphatidyl-ethanolamine (LC3-PE) (Abounit, Scarabelli and McCauley, 2012).

Atg5 plays an important role in the development of the autophagosomal membrane and deletion of this gene conventionally results in disruption of the autophagosome (Mizushima *et al.*, 2001). Atg7 and Atg10 conjugate Atg5 to Atg 12. The Atg12-Atg5 complex localizes the isolation membrane or phagophore, which is essential for recruitment of LC3 and maturation of the membrane into an autophagosome. The membrane lengthens (**elongation**) and **matures** into a cup-shaped isolation membrane, and LC3 is being conjugated to PE with Atg3. LC3-PE is subsequently recruited to the membrane in an Atg5-dependent manner (Mizushima *et al.*, 2001; Gustafsson and Gottlieb, 2008a).

Conjugated Atg5–Atg12 complexes pair with Atg16L to form a complex that associates with the exterior membrane of the phagophore (Ohsumi, 2001). The Atg5–Atg12–Atg16L complex is also thought to induce curvature into the growing phagophore through asymmetric recruitment of processed LC3B, also known as LC3II (Kadowaki and Karim, 2009; Glick, Barth and Macleod, 2010).

Beclin 1 (Atg6), in conjunction with these two conserved protein conjugation systems, are therefore necessary for the **formation, nucleation, elongation** and the **maturation** of the autophagosome (Ohsumi, 2001; Gustafsson and Gottlieb, 2008a).

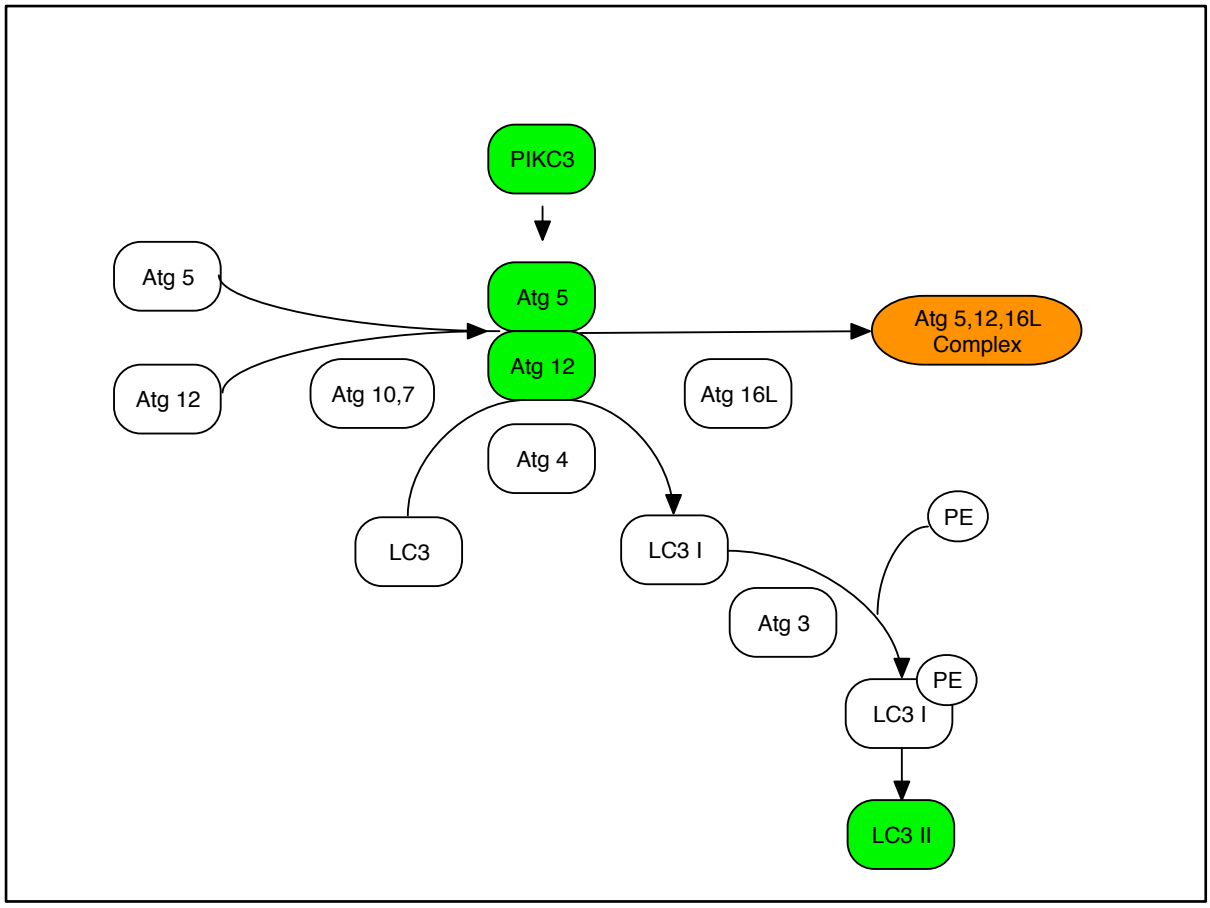


Figure 1.6: Mechanistic aspects of autophagosome formation and maturation

The protein systems Atg12-Atg5-16L complex in combination with LC3-PE, are responsible for the formation, nucleation, elongation and the maturation of the autophagosome. Abbreviations: Atg: autophagy related gene, PIKC3: Phosphatidylinositol kinase complex class three, LC3: light chain 3, PE: phosphatidylethanolamine.

c. Cargo recognition/selection:

Autophagy was initially described as a random, non-selective process because it appeared to engulf cytosol indiscriminately (Eskelinen, 2008b). We now know that the turnover of damaged organelles, removal of protein aggregates, and elimination of intracellular pathogens, are a selective and tightly regulated processes that requires cargo recognition (Stolz, Ernst and Dikic, 2014).

The molecular machinery for selective autophagy must ensure efficient recognition and sequestration of the cargo within the autophagosomes. There is evidence that the phagophore membrane interacts selectively with protein aggregates and organelles.

The composition of the outer and inner autophagosomal membranes seems to be different. At this stage only LC3 has been identified on the autophagosomal inner

membrane, (Kabeya *et al.*, 2000) where it acts as a receptor for p62 (also called sequestosome 1, SQSTM1) (Bjorkoy *et al.*, 2005; Mizushima, 2007). This LC3 'receptor' interacts with 'adaptor' molecules on different targets (e.g. protein aggregates and pathogens). The best-characterized adaptor molecule is p62, a multi-functional molecule that binds directly to LC3 and promotes the turnover of protein aggregates (Glick, Barth and Macleod, 2010). p62/SQSTM1 is preferentially *degraded* by autophagy and markedly accumulates in autophagy-deficient cells (Bjorkoy *et al.*, 2005; Mizushima, 2007).

Cargo selectivity is therefore being achieved by the autophagosomal membrane receptors (He and Klionsky, 2009; Stolz, Ernst and Dikic, 2014; Zaffagnini and Martens, 2016). Sciaretti *et al*. (2018) differentiates between mitophagy, lipophagy and ER-phagy to demonstrate cargo selectivity in autophagy (Sciarretta *et al.*, 2018). Mitophagy is dedicated to selective sequestration and degradation of mitochondria, during lipophagy (which is activated following lipid overload) autophagy selectively degrades lipid droplets (Mizushima and Komatsu, 2011), and ER selective ER-phagy mediates the elimination of burdened ER (Khaminets *et al.*, 2015).

d. <u>Autophagsome-lysosome fusion:</u>

Following above, the next step in this self-degradative process is fusion of the outer membrane of the autophagosome with a lysosome to form an 'autolysosome' or 'autophagolysosome'. This is done to create an acidic structure where lysosomal proteases can degrade the inner autophagosomal membrane and its cytoplasm-derived content (Mizushima, 2007).

This process of fusion is mediated by three soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein receptor (SNARE) proteins (STX17, SNAP29, and VAMP8), through a process dependent on Atg14 (Nair *et al.*, 2011; Diao *et al.*, 2015).

The autophagosome lysosome fusion takes place with the assistance of lysosomal-associated membrane protein 1 and 2 (LAMP-1 and 2), the small GTPase Ras related protein 7 (Rab7) and an ATPase called SKD1 (Tanida *et al.*, 2005; Thapalia, Zhou and Lin, 2014). Inactivation of LAMP-2 is associated with Danon disease in humans, a condition that causes cardiomyocyte hypertrophy and accumulation of autophagosomes in heart muscle (Tanaka *et al.*, 2000; Glick, Barth and Macleod,

2010) (Refer to Figure 1.7).

Another transcriptional mechanism playing a role in promoting autophagosome formation, autophagosome-lysosome fusion, and cargo degradation during starvation is transport of transcription factor EB (TFEB). TFEB dependent autophagy is controlled by ERK2 and mTOR, and is responsible for the reported cardiac protective effect against reperfusion injury secondary to intermittent fasting (Godar *et al.*, 2015).

e. Cargo breakdown followed by release of the degradation products: (Refer to Figure 1.7)

Following the autophagosome lysosome fusion, the proteolytic degradation of the inner vesicle along with its engulfed molecules occurs.

This depends on a series of lysosomal acid hydrolases, including Cathepsins B, D and L (Levine and Kroemer, 2008). The small molecular end-products of degradation (amino acids, fatty acids, nucleic acids, glucose and autophagy related gene proteins) are then released into the cytosol for reuse and recycling. Atg 2, Atg 9 and Atg 18 are responsible for Atg protein disassembly (Levine and Yuan, 2005; Mizushima, 2007; Glick, Barth and Macleod, 2010; Thapalia, Zhou and Lin, 2014).

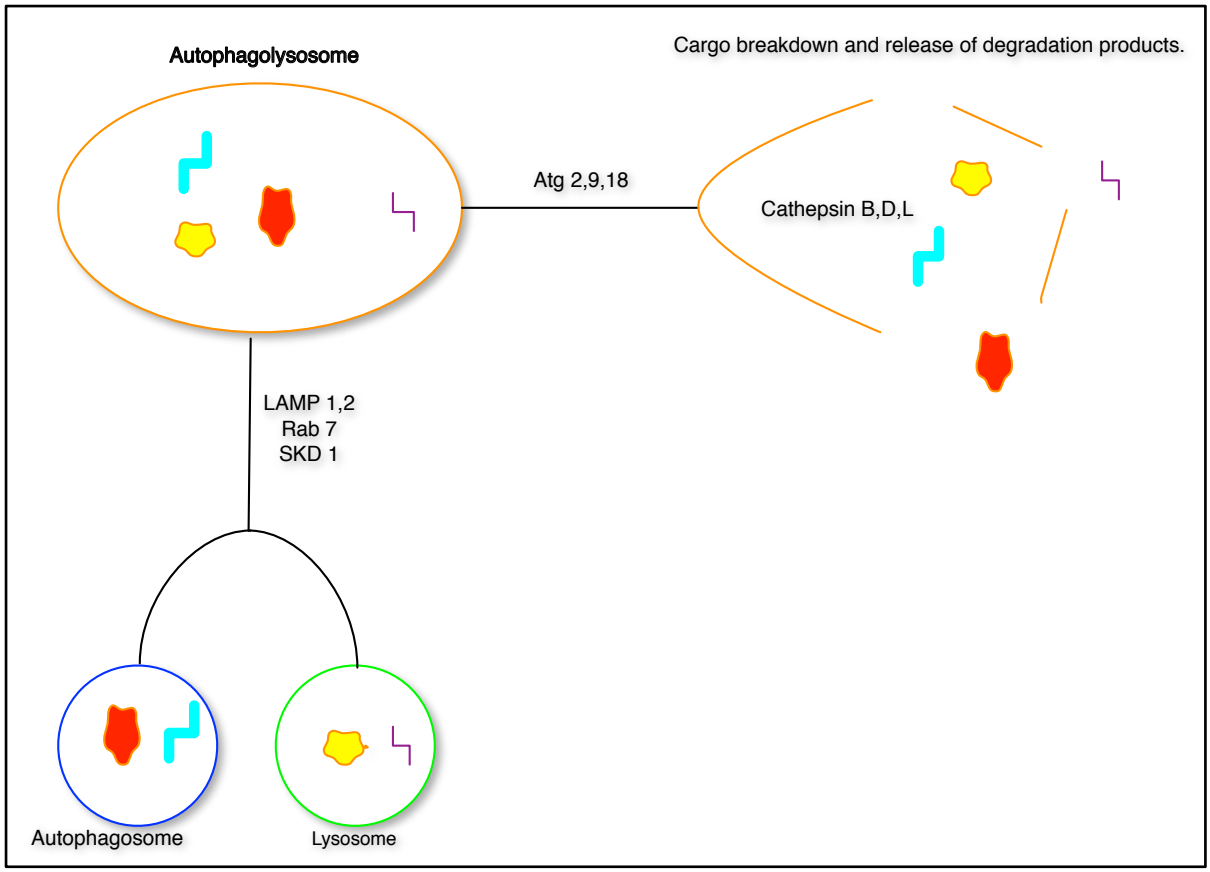


Figure 1.7: Autophagosome formation, cargo breakdown and release of degradation products

Autophagosome lysosome fusion take place with the assistance of LAMP-1 and 2, Rab7 and SKD1. Following the fusion, the proteolytic degradation of the inner vesicle along with its engulfed molecules (represented by the coloured small figures) occurs depending on Cathepsins B, D and L. Abbreviations: Atg: autophagy related gene, LAMP: lysosomal-associated membrane protein, SKD: suppressor of potassium transport growth defect.

1.5. ALTERNATIVE PATHWAYS

Two alternative autophagy pathways have been described:

- The Atg5/Atg7-independent pathway, and
- The non-canonical (Beclin-independent) pathway (Juenemann and Reits, 2012).

Non-canonical autophagy involves only a subset of Atg proteins for the formation of autophagosomes. It does not require the normal hierarchical participation of all the Atg proteins – as is the case of canonical autophagy (Codogno, Mehrpour and Proikas-Cezanne, 2011).

The hallmarks and important differences, with regard to this study, are summarised in Table 1.2.

Table 1.2: Key characteristics of, and the main differences between the conventional and alternative autophagic pathways

Autophagic pathways	Membrane source	Dependent on	Independent from	3MA inhibition	Rapamycin induction
Conventional pathway	ER	Atg5, LC3 and Beclin	-	Yes	Yes
Atg 5/Atg7 independent pathway	Golgi network and late endosomes	Rab9 and Beclin	Atg5 and LC3	Yes	No
Beclin-independent pathway	Unknown	Atg5 and LC3	Beclin	No	Yes

(Juenemann and Reits, 2012).

Conventional autophagy clearly depends on Atg5 and Atg12 and is associated with an increase in LC3B. Shimizu *et al.* (2010) attempted to determine if macroautophagy is completely abolished in Atg5-deficient cells. In their study, during which they used wild-type and Atg5-deficient mammalian cells, they concluded that two macro-autophagy systems exist: the conventional Atg5-dependent system and the "alternative" Atg5-independent system. Although both these pathways lead to the breakdown of damaged cytoplasmic organelles during autophago-lysosome production, the following differences exist (Shimizu, Arakawa and Nishida, 2010):

- The alternative pathway is controlled by Ulk1 protein and not by Atg5/Atg7.
- The main trigger (for the alternative autophagy pathway) is not Rapamycin and nutrient deprivation (as for the conventional pathway), but rather by stress causing DNA damage.
- The alternative pathway is independent of Atg5 expression and
- There is an absence of LC3A to B alteration.
- This new pathway further depends on Rab9 for the fusion between the trans Golgi network and late endosomes (Shimizu, Arakawa and Nishida, 2010).

The Rab proteins play an important role as a vesicular trafficking protein in autophagy. Rab1, 5, 7, 8B, 9, 11, 23, 24, 25, 32 and Rab33B are all essential in the conventional

autophagic pathway, whereas Rab9 is required for the alternative pathway (Ao, Zou and Wu, 2014). Rab9 is the most probable replacement for the Atg5-associated function in this alternative autophagic pathway (Shimizu, Arakawa and Nishida, 2010).

1.6 MEASUREMENT OF AUTOPHAGY

Different options to measure autophagy exist, the two main approaches include quantification of autophagy-/lysosome-dependent degradation products (proteins) and direct observation of autophagy-related structures (Yoshii and Mizushima, 2017). The detection of autophagic proteins by western blot or fluorescence studies, together with electron microscopy for autophagosome formation, have thus been the mainstays for the detection of autophagy (Barth, Glick and Macleod, 2010).

Transmission electron microscopy (EM) is valuable because it is the only measuring tool that reveals the morphology of autophagic structures, it also display these structures in their natural environment and position in relation to other cellular components (Klionsky *et al.*, 2016).

The process of maturation, from the phagophore to the autolysosome and the autophagolysosome, is a dynamic and continuous process which can be followed by EM. Cytoplasmic components enclosed in a double-membrane vesicle is the hallmark of an autophagosome on EM (Moulis and Vindis, 2017). Following sequestration of the cytoplasmic organelles and the formation of the autophagosome, it will fuse with a lysosome to form an autophagolysosomes - this can be characterised as a single-membraned structure containing degraded components (Eskelinen, 2008b, 2008a).

There are however also problems in utilising EM. It is time consuming, needs technical expertise (for fixation, sectioning and staining) and considerable experience to be able to identify autophagic structures. The difficulty lies in the fact that many subcellular components may be mistaken for autophagic structures (Klionsky *et al.*, 2016). The most significant critique against EM is that it cannot be used as a reliable measure to objectively quantify autophagic activity. Efforts have been made to quantify autophagy by measuring the “ratio of early to late autophagic compartments or autophagic volume as a percentage of cytoplasmic volume” (Ylä-

Anttila, Vihinen, Jokitalo and E. L. Eskelinen, 2009), this is also very interpreter depend. EM is therefore an important qualitative tool to monitor steady-state levels of autophagy and to gain insight into the structural inter-relationship between autophagic and other cellular organelles (Barth, Glick and Macleod, 2010).

Western blotting and fluorescence studies can be used in a quantitative autophagic measuring capacity (Klionsky *et al.*, 2008; Rubinsztein *et al.*, 2009)

Quantifying autophagy related protein activity can give insight in different stages in the process of autophagy. ULK1 complex embodies the most "upstream" autophagy factors and represents initial autophagosome formation. ULK1 complex inhibition will thus result in the inability to initiate autophagy (Itakura and Mizushima, 2010). Beclin1 also signals the onset of autophagy, (Cao and Klionsky, 2007) and many researchers use this protein as a way to monitor autophagy (Klionsky *et al.*, 2016). The formation of the isolation membrane and autophagosome can be tracked by both ULK1 and LC3 activity. LC3 activity will furthermore represent the fusion between the autophagosomes and lysosome. LC3 II it is the only well-described protein that is specifically localized to the different autophagic structures throughout the autophagic process (Nakatogawa *et al.*, 2009). P62/SQSTM1 can be used in combination with LC3 to represent autophagosome and lysosome function as well as the degradation of the autophagolysosome, p62 activity can thus serve as an index of autophagic degradation (Klionsky *et al.*, 2016). Rab9 and DRP can be used to investigate the alternative pathways of autophagy.

All the proteins used in immunoblotting, how to interpret them and their role as well as potential pitfalls in terms of the measurement of autophagic activity are being discussed in Chapter 7, Section 7.4.

Immunofluorescence is another invaluable technique used to quantify autophagic activity. This technique makes use of visualization of the subcellular organelles, by specific recognition of the antibody to the endogenous protein itself. Autophagy, as discussed, is a catabolic pathway in which dysfunctional organelles and cellular components are degraded via lysosomes. During this process, cytoplasmic LC3 translocate to autophagosomal membranes. Therefore, cells undergoing autophagy

can be identified by visualizing fluorescently labeled (for example) LC3. (Li *et al.*, 2019).

Limitations associated with this technique include the following: Counting the green fluorescent protein (GFP) -positive punctate structures is a laborious and subjective task, over-expressed GFP–LC3 can be incorporated into protein aggregates that is independent of autophagy, transfection procedures (to introduce exogenous GFP–LC3) may has induce autophagy, GFP–LC3 is sensitive to an acidic pH and will stop to fluoresce once autophagosome lysosome fusion takes place, resulting in the inability to look at end-stages of autophagy (Kimura, Noda and Yoshimori, 2007).

Measuring autophagy in a meaningful way requires an analysis of the rate of autophagic flux (the rate of autophagosome delivery to lysosomes, followed by degradation) as opposed to a snapshot look at autophagy at any one static point during the autophagic process (Barth, Glick and Macleod, 2010). The above-mentioned methods are therefore not sufficient as an isolated approach. High autophagosome content could reflect an increased in formation *or* a decreased clearance of autophagosomes (Hamacher-Brady, Brady and Gottlieb, 2006b). The determination of *autophagic flux* is therefore a more reliable representation of autophagic activity (Brady *et al.*, 2007; Iwai-Kanai *et al.*, 2008). Autophagic flux can be studied by analysing the amount of autophagosomes at steady state and after the inhibition of lysosomal degradation (Brady *et al.*, 2007). Chloroquine, to inhibit autophagosome lysosome fusion, its alternatives as well as autophagic flux are being referred to again in Section 7.5.3 (Gustafsson and Gottlieb, 2008a; Gottlieb, Finley and Mentzer, 2009).

CHAPTER 2
MYOCARDIAL ISCHEMIA

2.1. INTRODUCTION

Ischemic heart disease (IHD), which includes acute myocardial infarction (AMI), is the leading cause of morbidity and mortality in the world, (Mozaffarian *et al.*, 2015) and the principal contributor to the burden of disease (Anderson and Morrow, 2017; Neri *et al.*, 2017). In the United States alone, approximately one million people per year suffer from a myocardial infarction (Turer and Hill, 2010; Frank *et al.*, 2012).

Angina Pectoris is a very well-known form of presentation of myocardial ischemia in humans, and was first described by John Warren in 1812 (Warren, 1812; Nabel and Braunwald, 2012).

Myocardial ischemia (from the Greek *isch*-, restriction and -*hema*, blood) develops when there is an imbalance between oxygen demand and supply, resulting in damage or dysfunction of the cardiac tissue (Frank *et al.*, 2012).

The end result of ischemia is an infarction which is also known as myocardial necrosis. Evidence to support this diagnosis includes clinical symptoms (like chest pain), acute electrocardiograph (ECG) findings suggestive of ischemia, and/or new wall motion abnormalities in a myocardial segment (Thygesen, Alpert and White, 2007; Ambrose and Singh, 2015).

2.2. DIFFERENT FORMS OF PRESENTATION OF MYOCARDIAL ISCHEMIA

Ischemia is caused by an insufficient supply of blood to an organ. This can be responsible for a cerebrovascular incident or a transient ischemic attack (brain ischemia), mesenteric ischemia (intestinal ischemia), a gangrenous foot (ischemia of the lower limbs) and ischemic optic neuropathy (eye ischemia).

The clinical presentation of *myocardial ischemia* in humans includes:

- Angina, which might be
  a) Symptomatic, in the form of stabile -, unstable - or Prinzmetal angina.
  b) Asymptomatic, also in the form of stabile -, unstable - or Prinzmetal angina.

- Myocardial infarction
  a) ST segment elevation infarction (STEMI).
  b) Non-ST segment elevation infarction (NSTEMI).
  c) Sudden death secondary to cardiac arrhythmias.
  d) Shortness of breath and pulmonary oedema secondary to left ventricular failure.

The extent of the coronary occlusion, the volume of the affected ischemic myocardium, degree of collateral circulation, pre-existing metabolic rate, genetic factors, and the intrinsic survival capacities of the myocytes will determine the specific clinical presentation (Ferrari *et al.*, 2017).

2.3. RISK FACTORS ASSOCIATED WITH ISCHEMIC HEART DISEASE.

A risk factor is defined as a "measurable characteristic that is causally associated with increased disease frequency, and that it is a significant independent predictor of an increased risk of presenting with the disease" (O'Donnell and Elosua, 2008).

Table 2.1: Risk factors associated with ischemic heart disease

Modifiable	Non-modifiable
High total serum cholesterol High LDL-cholesterol Low and high HDL-cholesterol High triglycerides	Family history of ischemic heart disease
Hypertension and left ventricular hypertrophy	Age
Diabetes	Gender (male)
Obesity	Post-menopausal
Physical inactivity	
Smoking	
Excessive alcohol	
Excessive stress	
Diet	

Source: Mahmood *et al*., 2014.

The role of diet in terms of a cardiovascular risk factor is controversial and the real culprit – sugar or fat – is still being debated.

The most recent study investigating the role of diet on ischemic heart disease, The Prospective Urban Rural Epidemiology (PURE) study (which included 135 335 individuals), reported the following: High carbohydrate intake and a higher risk of mortality are associated with each other, whereas higher total fat was related to a lower mortality. Different types of fat were not associated with cardiovascular disease, myocardial infarction or cardiovascular disease mortality. It was advised that global dietary guidelines should be reconsidered (emphasizing low carbohydrate, rather than low fat) following this study that lasted from 2003 to 2013 (Dehghan *et al.*, 2017).

2.4. ATHEROSCLEROSIS

All of above-mentioned risk factors (according to the injury hypothesis) will lead to a degree of coronary atherosclerosis (in humans) with resulting damage to the endothelium which is ultimately responsible for the different forms of presentation of myocardial ischemia.

Conventionally, the initiating factor for atherosclerosis is considered to be high levels of low density lipoprotein (LDL) (Schachter, 1997). Half of atherosclerotic related deaths however occur in patients without obvious hyperlipidemia. Non-traditional risk factors, with the emphasis on inflammation, are now considered to play an important role (Libby, Ridker and Maseri, 2002). The importance of inflammation in IHD was demonstrated by a clinical trial making use of canakinumab, an IL-1β–targeting antibody. In this trial, highly-sensitivity C-reactive protein and interleukin-6 levels were significantly reduced by canakinumab and the higher dosage resulted in a significantly reduction in recurrent cardiovascular events (Ridker *et al.*, 2017). The anti-inflammatory effect (not the lipid lowering effects) of statins on the formation of atherosclerotic plaque and the function of endothelial cells act as further support to the important role of inflammation in IHD (Diamantis *et al.*, 2017).

The first step in the initiation of atherosclerosis is the build-up of LDL particles in the arterial intima (Libby, Braunwald and Zipes, 2001). These lipoproteins then undergo oxidation and glycation, (Libby, 2000; Libby, Ridker and Maseri, 2002) which have potent pro-inflammatory properties, contributing to atherosclerotic plaque development (Levin and Coetzee, 2007).

Normal endothelium usually has a low affinity for the adhesion of nearby leucocytes.

Oxidized and glycated LDL however trigger the release cell adhesion molecules which are responsible for an increase in the expression of immunoglobulins (intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1)) (Libby, 2000; Libby, Braunwald and Zipes, 2001; Libby, Ridker and Maseri, 2002). This increase in cell adhesion molecule release facilitates the bond between monocytes and T lymphocytes to the endothelial cells. Monocyte chemoattractant protein 1 (MCP-1) (released secondary to inflammatory processes) thereafter facilitates their entry into the intima (Libby, 2000; Libby, Braunwald and Zipes, 2001; Libby, Ridker and Maseri, 2002).

Monocytes change into macrophages after arrival into the intima. The macrophages are being activated by macrophage colony stimulating factor (M-CSF), which increases the expression of surface scavenger receptors (Libby, 2000; Libby, Braunwald and Zipes, 2001). Normal (LDL) receptors are subjected to a negative feedback inhibition, which is activated when the cell has sufficient cholesterol (from LDL) to satisfy its metabolic needs (Libby, Braunwald and Zipes, 2001). *Scavenger receptors*, however, facilitate a disproportionate lipoprotein uptake. The macrophages then store and oxidize the lipoproteins, after which they leave the arterial wall – in an attempt to cleanse the blood vessels (Libby, 2000). Macrophage-monocytes containing high levels of LDL have a typical vacuolated appearance and are termed “foam cells or lipid laden macrophages” (Libby, 2000; Libby, Braunwald and Zipes, 2001). A foam cell collection is known as a *fatty streak*, which is the foundation of an atherosclerotic plaque. The endothelium covering a fatty streak, although anatomically intact, is already dysfunctional (Levin and Coetzee, 2007).

New research however contradicts the above role of foamy macrophages. A recent study regarding the different roles of nonfoamy versus foamy macrophages were done by Kim *et al*. (2018). Making use of two RNA sequencing approaches, they discovered that nonfoamy macrophages play the dominant role in the immune response. The foamy macrophages, referred to earlier, were mainly involved in lipid processing (and not inflammation, as previously thought). The foamy macrophages furthermore demonstrated less IL-1β expression (and thus a smaller inflammatory role) than what was found in the nonfoamy macrophages (Kim *et al.*, 2018).

Smooth muscle cells (from the tunica media) will then gradually migrate into, and

multiply in, the arterial intima. Smooth muscle cells generate extracellular matrix macromolecules (collagen, proteoglycans and elastin) responsible for the upkeep and integrity of the arterial wall. An increase in the production of cytokines and growth factors (during inflammation) will however stimulate an overexpression of extracellular matrix proteins by the smooth muscle cells, which is responsible for the *fibrous cap* that covers the more mature atherosclerotic plaques (Levin and Coetzee, 2007).

The thickness of the fibrous cap is determined by the balance between the synthesis of the extracellular matrix molecules vs their breakdown by proteolytic enzymes (matrix metalloproteinases). Matrix metalloproteinases (MMPs) are being produced by foam cells secondary to inflammatory stimuli such as TNF alpha, IL-1, oxidized LDL and activated T lymphocytes (Falk, Shah and Fuster, 1995; Libby, Ridker and Maseri, 2002). Inhibitors of Matrix metalloproteinases, otherwise known as Tissue Inhibitors of Metalloproteinases (TIMPs), can prevent extracellular matrix macromolecules breakdown (Libby, Braunwald and Zipes, 2001).

Inflammation may contribute to the death of smooth muscle and foam cells (Libby, Braunwald and Zipes, 2001). The resulting necrosis is responsible for the initiation of a necrotic lipid core in the atherosclerotic plaque (Libby, 2000). A *mature plaque* therefore comprises a collagen rich fibrous capsule surrounding the lipid rich, necrotic core (Levin and Coetzee, 2007).

Endothelial function in atherosclerosis

Endothelial dysfunction refers to (Cai and Harrison, 2000):

- The loss of endothelium dependent vasodilatation,
- Abnormal anticoagulation,
- Compromised modulation of vascular growth and
- Dysregulation of vascular remodeling.

Endothelium normally expresses nitric oxide synthase (eNOS) in response to stimuli such as shear stress (Kinlay, 2005). Oxidized LDL causes a reduction in the production of nitric oxide (Ray and Cannon, 2004). This nitric oxide deficiency contributes to platelet aggregation, impaired endothelium dependent vasodilatation, hypertension and enhanced leukocyte aggregation (Cai and Harrison, 2000). The

atherosclerotic endothelium therefore contributes to a prothrombotic, vasoconstrictor environment which promote an increased likelihood of occlusive thrombus formation during plaque rupture (Ray and Cannon, 2004; Levin and Coetzee, 2007).

2.5. PATHOPHYSIOLOGY OF MYOCARDIAL ISCHEMIA

Atherosclerosis, a chronic disease, has an unique anatomical morphology (either eccentric or concentric lesions) which is responsible for pathophysiology via different mechanisms (Libby, 2000; Libby, Braunwald and Zipes, 2001).

There are two distinct mechanisms responsible for myocardial ischemia:

- Acute coronary syndrome and
- Prolonged myocardial oxygen supply-demand imbalance in the presence of stable coronary artery disease (CAD) (Landesberg *et al.*, 2009).

2.5.1. Acute coronary syndrome

Acute coronary syndrome will occur secondary to *vulnerable (unstable) plaque rupture* or endothelial ulceration, and is essentially a *platelet associated* disease process clinically consisting of unstable angina, NSTEMI and STEMI (Landesberg *et al.*, 2009) (Refer to Figure 2.1).

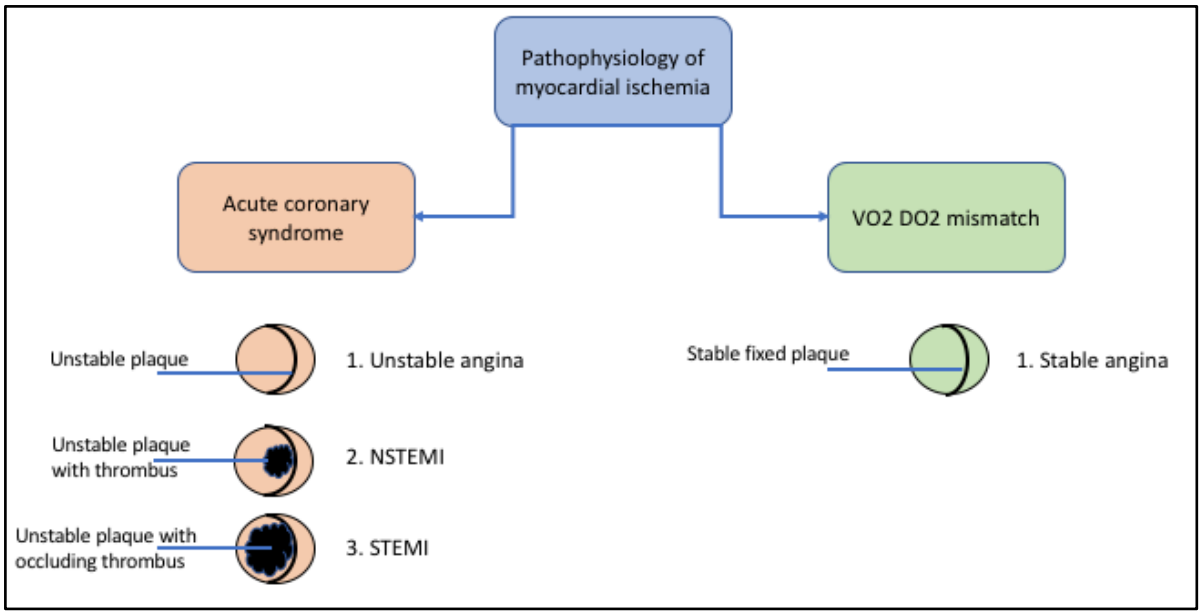


Figure 2.1: Pathophysiological mechanisms responsible for cardiac ischemia

Abbreviations: VO_2: oxygen consumption, DO_2: oxygen delivery, MI: myocardial infarction.

The factors contributing to this plaque instability include (Gertz and Roberts, 1990; Priebe, 2005):

a) Risk factors (most importantly, inflammation), (Casscells, Naghavi and Willerson, 2003)

b) The heterogeneity of plaque histology (how vulnerable the plaque is), (Maseri and Fuster, 2003) and

c) The physical forces to which plaques are exposed to (Falk, Shah and Fuster, 1995).

The following two factors determine if a plaque can be defined as "*vulnerable*" – and therefore has a high susceptibility of rupture (Naghavi *et al.*, 2003, 2006).

1. A thin fibrous cap of less than 65 to 150 microns.
2. A necrotic lipid core, which involves more than 40% lesion (Falk, Shah and Fuster, 1995; Priebe, 2004).

The above combination implies a high wall stress in the fibrous cap, and plaque rupture is known to happen at the area of maximal wall stress (Falk, Shah and Fuster, 1995; Levin and Coetzee, 2007).

The thrombotic environment, plaque volume and composition, the amount of luminal narrowing and the extend of the fibrous cap rupture, will all act together to determine the degree and duration of symptoms secondary to an ischemic event (Srikanth and A. Ambrose, 2012).

2.5.2. Myocardial Oxygen Supply-Demand Imbalance (DO_2-VO_2 imbalance)

The other potential mechanism responsible for myocardial ischemia occurs when oxygen delivery is not sufficient to meet the oxygen demands.

The heart is an obligate aerobic organ, entirely dependent on coronary perfusion for an uninterrupted oxygen supply. In the normal state, myocardial oxygen demand is almost always met by myocardial oxygen delivery. The normal myocardium therefore does not become ischemic during an increase in oxygen consumption (as occurs

during maximal exercise testing) (Detry J-M, 1996). Coronary blood flow may increase up to fourfold to compensate for the increase in oxygen consumption during exercise (Lombardo *et al.*, 1953). There is an almost perfect linear relationship between coronary blood flow and myocardial oxygen consumption (Knabb *et al.*, 1983). It is this match that exists between myocardial oxygen consumption (VO_2) and oxygen delivery (DO_2) that remains central to our understanding of myocardial ischemia (Crossman, 2004).

The obligatory increase in coronary blood flow is required because of the high oxygen extraction under normal resting conditions (coronary sinus oxygen saturation at rest is around 25–30%, thus the myocardium extracts the majority of oxygen from the coronary blood). The coronary circulation can therefore be defined as a low flow – high extraction regional circulation in contrast with, for instance, renal blood flow which is a high flow and low extraction regional circulation. Coronary circulation is in contrasts with total cardiac output and whole body circulation, where an increase in whole body oxygen consumption can be compensated for, in part, by increasing oxygen extraction (Crossman, 2004).

Determinants of myocardial oxygen consumption and oxygen delivery

The major determinants of **myocardial oxygen consumption** (**VO_2)** are myocardial contractility and ventricular wall tension (and therefore left ventricular size) (Sonnenblick and Braunwald, 1968; Crossman, 2004). Ventricular wall tension, as defined by Laplace's law, is the product of the transmural pressure (difference between intra-ventricular pressure and the intra-thoracic pressure) and the radius of the ventricle, divided by myocardial wall thickness (Basford, 2002).

Under normal physiological conditions (when metabolic substrates are in sufficient supply) oxygen consumption will be determined by the metabolic needs of tissues.

The equation for oxygen delivery can be used to summarize the major determinants of **myocardial oxygen delivery (DO_2)**: Oxygen delivery to the myocardium (MDO_2) is determined by the product of arterial oxygen content (CaO_2) and the coronary blood flow (CBF).

$MDO_2 = CBF \times CaO_2$

The variables determining arterial oxygen content include oxygen saturation (SaO_2), haemoglobin (Hb), Hüfner's constant and the partial pressure of oxygen (PaO_2).

$CaO_2 = (SaO_2 \times Hb \times 1.39) + (0.0031 \times PaO_2)$

Lastly, coronary blood flow equals coronary perfusion pressure (CPP) divided by the coronary vascular resistance (R). Coronary perfusion pressure equals the difference between aortic diastolic pressure (AoDP, the driving pressure) and the downstream pressure. The latter is, in correct physiological parlance, the tissue pressure (Zo) which is in the order of 25 mm Hg in the normal heart and higher than the left ventricular end diastolic pressure (LVEDP, or the tissue pressure). However, for practical reasons LVEDP is conventionally used because of our inability to reliably obtain the correct Zo in clinical practice.

CBF = CPP/R
CPP =AoDP – LVEDP

Coronary perfusion to the left ventricle occurs mainly in diastole and the diastolic time (i.e. heart rate) is therefore of crucial importance to sufficient left ventricular coronary perfusion (Homoud, 2008). The right ventricle perfusion pattern follows the aortic pressure i.e. it occurs mainly in systole. Flow across the myocardium largely depends on the pressure gradient between the aortic root (the ‘‘coronary driving pressure’’) and the tissue pressure (Zo) (Schelbert, 2010).

The different components of coronary vascular resistance (R) are viscosity (η), vessel length (l) and vessel radius (r)), as summarized by the Hagen-Poiseuille equation, which states that the resistance to flow depends on the fourth power of the vessel diameter (Schelbert, 2010; Gooch, 2011).

$R = 8\eta l/\pi r^4$

Coronary vessels can be divided into epicardial (conductance) vessels, pre-capillary and microvascular vessels. Atherosclerosis mostly targets the epicardial vessels, while flow resistance occurs mainly in the resistance vessels (pre-capillary and microvascular vessels). The increase in coronary blood flow following an increase in oxygen demand (autoregulation) is a result of *resistance vessel* dilatation (Homoud,

2008).

In summary, coronary blood flow is directly dependent upon the difference between the driving pressure (diastolic blood pressure) and the left ventricular end diastolic pressure (tissue pressure) and inversely proportional to the coronary vessel resistance (Crossman, 2004; Homoud, 2008).

2.5.3. Pathophysiology of stable angina

A mentioned before, fibrous cap fissures and rupture are not always accompanied by significant clot (thrombus) formation. It may (only) stimulate a fibrotic response with subsequent slowly enlarging fibrous tissue, which will result in a decrease in the lumen of the vessel. This reduction in the coronary artery diameter is responsible for the increase in resistance in the coronary artery, with a resulting reduction in coronary blood flow (Levin and Coetzee, 2007).

Coronary flow reserve refers to the potential increase in coronary perfusion to accommodate an increase in metabolic needs. The increase in coronary perfusion will be the consequence of intrinsic metabolic autoregulation. Coronary flow reserve can therefore be used to refer to the difference between basal and the maximum coronary blood flow (Crossman, 2004).

Taking above into consideration atherosclerosis may have a twofold effect on coronary blood flow.

1. It may be responsible for coronary artery stenosis giving rise to an increase in resistance and therefore a decrease in coronary flow.
2. The increase in alpha adrenergic receptors in atherosclerotic coronary arteries, (Heusch, 1990) in combination with the inability to release vasodilating agents also prevent autoregulation. The coronary flow reserve will therefore be limited secondary to the failure to vasodilate and to reduce the vascular resistance (due to endothelial dysfunction) (Zeiher *et al.*, 1991).

In these patients (with endothelial dysfunction) an increase in oxygen consumption cannot be matched by a sufficient increase in myocardial blood flow, therefore potentially eliciting myocardial ischemia (Deanfield *et al.*, 1983).

Ischemia during above-mentioned circumstances is therefore the consequence of the coronary vessel's inability to match the increase in oxygen demand. The variability in terms of angina symptoms is likely to originate from the inconsistencies in coronary flow reserve rather than variability of myocardial oxygen consumption (Crossman, 2004).

In relating **Ohm's Law** to the flow of fluid: Flow (Q) will equal the pressure difference (P1 – P2); sometimes called driving pressure, perfusion pressure, or pressure gradient, divided by the resistance to flow (R) supplied by the blood vessel (Guyton and Hall, 2006).

This hemodynamic relationship can thus be summarized by: Q = (P1 – P2)/R

Once R increases (arterial diameter decreases) or P1 decreases (hypotension), P2 will have to decrease (coronary vasodilatation must occur) in an attempt to maintain flow. Reductions of perfusion pressure (P1) are normally countered by coronary vasodilation (P2) (during autoregulation) in an attempt to maintain sufficient blood flow (Q).

A very prominent characteristic of a flow limiting coronary stenosis is the marked decrease in coronary pressure distal to the stenosis (decrease in P2) (Bache, McHale and Greenfield, 1977). Rouleau *et al*. (1979) demonstrated that progressive reductions of perfusion pressure would result in maximal vasodilation of the subendocardial vessels (Rouleau *et al.*, 1979). Further decreases in coronary perfusion pressure (P1) will then be followed with a reduction of subendocardial flow as a direct function of pressure. Gallagher *et al*. demonstrated that "in the presence of a critical stenosis there is no coronary reactive hyperemic response because maximal vasodilation has already occurred" (Gould, 1978; Bache and Schwartz, 1982).

Flow in the setting of critical ischemia is therefore directly dependent on the coronary driving pressure and, thus, on the arterial blood pressure (P1), (Schelbert, 2010) since the coronary resistance is a constant and the vasodilatory response is maximal (Q α P1) (Coetzee, 1984).

2.5.4. Coronary artery spasm

Except for acute coronary syndrome (thrombosis) and prolonged VO2 DO2 mismatch

in the presence of stable coronary artery disease one more cause for angina exists. This is known as prinzmetal angina, a cyclic syndrome that typically occurs at rest and is caused by vasospasm of (normal) coronary arteries rather than atherosclerosis (Prinzmetal *et al.*, 1959).

The pathophysiology of coronary artery spasm is multifactorial, but it appears that the most important causative factor for coronary spasm is an increased intracellular calcium concentration in combination with an increased calcium sensitivity (Yasue *et al.*, 2018).

2.6. CONSEQUENCES OF REDUCED BLOOD FLOW TO THE MYOCARDIUM

The consequences secondary to a reduction in myocardial blood flow will be determined by the duration as well as the severity of the compromised blood flow. Possibilities include hibernation, the spectrum of the ischemic cascade and myocardial necrosis.

2.6.1. Myocardial hibernation

Resting wall-motion abnormalities visualized in patients with coronary artery disease can improve upon revascularisation (coronary artery bypass). In 1978 Diamond *et al.* (1978) first described the hibernating myocardium as follows: “Reports of sometimes dramatic improvement in segmental left ventricular function following coronary bypass surgery, although not universal, leaves the clear implication that ischemic non-infarcted myocardium can exist in a state of functional hibernation” (Diamond *et al.*, 1978). This was followed by an article published by Rahimtoola in the 1980’s during which he described a patient group, with coronary artery disease, in whom chronic left ventricular dysfunction improved upon revascularization. He commercialized myocardial hibernation and described it as “a state of persistently impaired myocardial and left ventricular function at rest due to reduced coronary blood flow that can be partially or completely restored to normal either by improving blood flow or by reducing oxygen demand”(Rahimtoola, 1995; Camici *et al.*, 1997).

The term “hibernation” therefore implies an adaptive reduction in energy use (decrease in oxygen consumption) in the presence of reduced oxygen delivery (decrease in coronary blood flow). Oxygen delivery will therefore determine oxygen consumption in these circumstances, and not the other way around as normally seen.

The features that characterize hibernating myocardium include:

- A chronically reduced baseline myocardial blood flow, enough to cause a decrease in myocardial function.
- Ischemic consequences such as necrosis excluded.
- The ability to demonstrate remaining contractile reserve.
- Rapid (functional) recovery of the depressed myocardium following revascularization (Camici *et al.*, 1997).

This chronic, reversible, contractile dysfunction in patients with coronary artery disease might be explained by the following two hypotheses (Ferrari *et al.*, 2017):

- The chronic reduction in coronary blood flow (chronic ischemia with a decrease in oxygen delivery) results in an intracellular acidosis and a reduction in contraction (oxygen consumption). This allows for the maintenance of energy (oxygen demand and consumption) matching.
- The second hypothesis views hibernation as the product of repetitive ischemic insults, which are responsible for the maintenance of a chronic state of myocardial stunning (Ferrari and Hearse, 1997; Ferrari *et al.*, 2017). This concept was already suggested in 1982 by Braunwald and Kloner (Braunwald and Kloner, 1982) and more recently by Bolli (Bolli, 1992; Ghaleh, Shen and Vatner, 1996; Camici *et al.*, 1997).

Histological studies on chronically dysfunctional but viable (hibernating) myocardium demonstrated no necrosis but profound structural changes. The changes include: (1) A loss of contractile proteins (sarcomeres) in cardiomyocytes without a loss of cell volume, since the space is replaced by glycogen. (2) Increased numbers of small mitochondria next to the glycogen-rich zones. (3) Changes involving the nuclei where heterochromatin is found. (4) Significant loss of sarcoplasmic reticulum, which is being replaced by a messy organisation of reticular membranes. The sarcolemma also no longer projects T tubules into the cytoplasm (Borgers *et al.*, 1993; Maes *et al.*, 1994; Camici *et al.*, 1997).

The above changes resemble structural features of embryonic/fetal cardiomyocytes (Ausma *et al.*, 1995; Camici *et al.*, 1997).

The question is, are these histological changes always apparent in the hibernating myocardium? It seems unlikely that myocardial tissue with these morphological changes will be able to function immediately following revascularization – time will be required to regain an adequate amount of contractile material. The discrepancy between patients in terms of the normalization of myocardial function suggests a heterogeneity among the hearts of individual patients (Camici *et al.*, 1997).

2.6.2. Ischemia

The ischemic cascade, already described 20 years ago (Hauser *et al.*, 1985; Nesto and Kowalchuk, 1987), refers to a sequence of pathophysiological events that coincides with the increase in myocardial oxygen supply–demand imbalance.

A cascade is a sequence of events, each of which triggers the next. There is therefore literature stating that it is “unwise to describe the clinical manifestations of ischemia as a cascade”, seeing that the components of this cascade often occur out of sequence (Maznyczka *et al.*, 2015).

A transient interruption of coronary blood flow in a significant portion of the myocardium is poorly tolerated and may lead to the appearance of the above mentioned ischemic cascade which *usually* occurs in the following sequence (Nesto and Kowalchuk, 1987; Detry J-M, 1996; Maznyczka *et al.*, 2015):

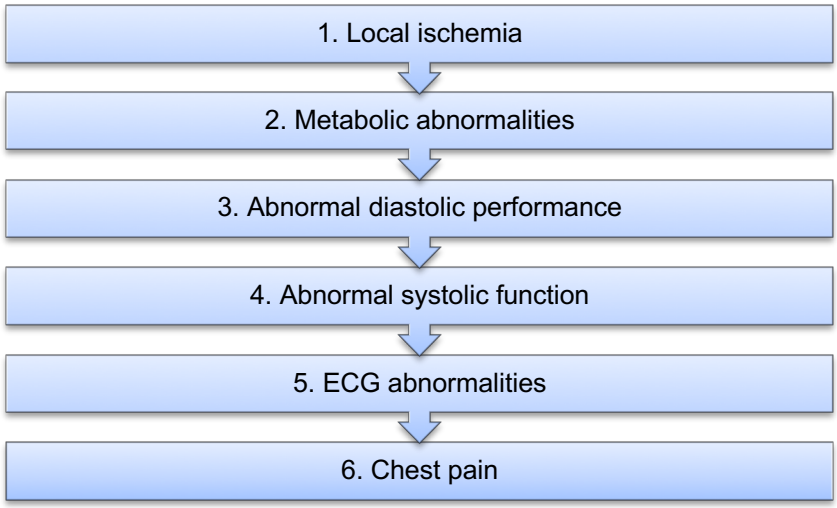


Figure 2.2: Schematic representation of the ischemic cascade (Detry J-M, 1996).

1 & 2. The deprivation of oxygen and nutrient supply, following ischemia, will results in a series of abrupt biochemical and metabolic changes within the myocardium. These changes trigger the ischemic cascade, the severity depending on the degree and the duration of the ischemic event.

Hypoxia will depress the mitochondrial oxidative phosphorylation process, which, in turn, will result in a decrease in aerobic ATP production. A compensatory switch to anaerobic glycolysis for ATP production will cause an increase in hydrogen ions and lactate production with subsequent intracellular acidosis (blood pH to <7.0) and inhibition of glycolysis (Thandroyen *et al.*, 1992). The initial increase in potassium efflux is secondary to an increased osmotic load due to the accumulation of metabolites and inorganic phosphate. The substantial decline in ATP inhibits the $3Na^+/2K^+$ATPase activity, causing a further decline of potassium and an increase in intracellular sodium. The intracellular accumulation of protons activates the Na^+/H^+ ion exchanger, which extrudes protons from the cell in exchange for sodium entry, exacerbating the intracellular sodium overload (responsible for cell swelling). The $2Na^+/Ca^{2+}$ ion exchanger is being triggered in response, resulting in an intracellular calcium overload as the cell tries to extrude sodium (Avkiran and Marber, 2002; Buja, 2005; Hausenloy and Yellon, 2013).

Late stage cardiomyocyte injury is characterized by progressive membrane damage. The mechanisms for *membrane damage* are secondary to (Thandroyen *et al.*, 1992; Buja, 2005):

- The sustained increase in cytosolic calcium that leads to phospholipase activation and phospholipid degradation followed by a release of lysophospholipids and free fatty acids.
- Impairment in mitochondrial fatty acid metabolism which result in the accumulation of free fatty acids, long-chain acyl CoA and acyl carnitine. These molecules in combination with the products of phospholipid degradation, are incorporated into membranes and impair their function.

- Generation of toxic oxygen species and free radicals from ischemic myocytes, endothelial cells and activated leukocytes. This may be responsible for fatty acid (located in the membrane phospholipids) damage (Buja, 2005).

All these events contribute to a gradual increase in membrane permeability.

The combination of membrane damage, intracellular electrolyte derangement and ATP exhaustion collectively contribute to the terminal event - a physical disruption of the sarcolemma of the swollen myocyte (Buja, 2005) followed by cell death (necrosis).

3. Diastolic dysfunction is the third step in the ischemic cascade. It is characterized by slow ventricular relaxation secondary to ATP exhaustion and the disruption in calcium homeostasis (Ferrari *et al.*, 2017).

4. Abnormal systolic function usually follows diastolic dysfunction. Poor systolic function may manifest as either regional wall motion abnormalities (local ischemia) or a global decrease in total cardiac performance expressed as a reduction in the ejection fraction. ATP depletion and the acidic environment inhibiting myofibril contracture, are the main contributing factors. Both a decrease in contractility (and therefore decreased ATP consumption) and increased anaerobic ATP production are, at the expense of myocardial function, cardio-protective mechanisms (Buja, 2005; Ferrari *et al.*, 2017).

5. Electrocardiogram changes in the form of ST-segment changes occurs next. This is usually in the form of (at least 1 mm from the baseline) horizontal depression.

6. The clinical manifestation of myocardial ischemia, in the form of angina pectoris, is normally the last phenomenon. This is indicative of severe ischemia in a given region of the myocardium (Buja, 2005).

These abnormalities progressively disappear on restoration of coronary blood flow. Twenty to 30 minutes of circulatory interruption (in dogs) will result in irreversible myocardial injury, progressing in a “wave front” from the sub-endocardium to the sub-epicardium (transmural injury) (Reimer *et al.*, 1977).

Coronary blood flow restored in time to prevent permanent myocardial injury, will still result in myocardial dysfunction, lasting for at least 24 hours. This phenomenon is known as stunning, and will be discussed in the next chapter (Detry J-M, 1996).

2.6.3. Myocardial necrosis (myocardial infarction)

The majority of myocardial infarcts (in humans) will be completed within 3 to 4 hours after the onset of severe ischemia. The extent of the myocardial necrosis, and therefore the determinants of the ultimate infarct size, will depend on the duration and severity of the flow interruption, the size of the myocardium at risk and the presence and degree of collateral blood flow (Detry J-M, 1996; Buja, 2005).

2.7. LONG TERM CONSEQUENCES OF MYOCARDIAL NECROSIS

Following myocardial infarction, the infarcted segment undergoes dilatation and thinning (expansion) without further myocyte loss. The resulting aneurysm formation will shift the hemodynamic forces from the infarcted to the healthier (non-infarcted) myocardial segments. The increasing stretch in these segments is an attempt to compensate for the loss in total cardiac inotropy, and is responsible for dilatation of this part of the heart. These changes, referred to as ***remodeling***, rely on the Frank-Starling law to augment cardiac output in the short term, but are detrimental in the long-term (Homoud, 2008).

“Remodeling” was coined by Hockman and Buckey in 1982 who used the term to described the replacement of infarcted tissue with scar tissue (Hochman and Bulkley, 1982). According to Janice Pfeffer the term refers to the gradual increase of the left ventricular cavity (Pfeffer, Pfeffer and Braunwald, 1985). A consensus statement from an international forum on cardiac remodeling defined cardiac remodeling “as a group of molecular, cellular and interstitial changes that clinically manifest as changes in size, shape and function of the heart resulting from cardiac injury” (Cohn, Ferrari and Sharpe, 2000). Cardiac remodeling is mainly a consequence of an inflammatory reaction, followed by scar formation at the infarction site and vascular remodeling and interstitial fibrosis in the “healthy” myocardium (Sun, 2008).

The clinical implications as a result of remodeling include: cardiac dysfunction (Kleinbongard *et al.*, 2011; Heusch *et al.*, 2014), malignant ventricular arrhythmias (Wang and Hill, 2010) and infarct expansion (Pfeffer MA, 1990).

The pathophysiological mechanism of cardiac dysfunction

The factors involved in this process of cardiac dysfunction include (Azevedo *et al.*,

2016):

- Cell death

Apoptosis, necrosis and autophagy are the main mechanisms involved in myocyte death.

Autophagy, as discussed before, is characterized by the destruction of superfluous or dysfunctional cytoplasmic proteins by lysosomes. A disturbance in protein balance may lead to the buildup of defective proteins, known as proteotoxicity. Autophagy therefore exerts a crucial role in the prevention of proteotoxicity. Evidence exist supporting the association between ventricular dysfunction and changes in autophagy (Burchfield, Xie and Hill, 2013).

The gradual loss of myocytes plays an important role in remodeling, and is therefore a target for future therapeutic interventions.

- Energy metabolism

The energy crisis resulting from the mismatch between oxygen supply and demand, is another mechanism contributing to cardiac dysfunction. This imbalance results in low myocardial ATP availability with resulting reactive oxygen species generation, oxidative stress and its effects (which will be discussed in the next chapter) (Azevedo *et al.*, 2013).

- Oxidative stress
- Inflammation (Mann, 2015)
- Collagen built-up.

Ninety-five percent of the myocardial interstitium consists of type I and type III collagen fibers (Zannad, Rossignol and Iraqi, 2010). Collagen plays an important role in the maintenance of cardiac structure and function, and the balance between collagen synthesis and degradation plays an important role in the remodeling process.

Collagen fibers are bound in such a way that they are resistant to degradation by most proteases. Metalloproteinases, for example, are enzymes with collagenolytic activity, and an increase in their activity is associated with changes suggestive of remodeling (Spinale, Janicki and Zile, 2013).

The excessive build-up of type III and type I collagen (which is harder, longer and more stable) was also detected in different models of cardiac injury. This increase in collagen (fibrosis) can be associated with impaired systolic function, reduction in coronary flow, malignant arrhythmias and a decrease in myocardial compliance with resulting diastolic dysfunction (Leask, 2015).

- Change in calcium transport system

Calcium transport (via L-type calcium channels, ryanodine receptor and calsequestrin) is an energy dependent process during cardiac systole and diastole (Luo and Anderson, 2013). Evidence implies that changes in the calcium transport system occur during ventricular remodeling which may contribute to systolic and diastolic cardiac dysfunction (Luo and Anderson, 2013; Feridooni, Dibb and Howlett, 2015).

- Neuro-hormonal activation

The two dominant hormonal systems involved in cardiac remodeling are the sympathetic system and the renin–angiotensin–aldosterone (RAA) system. Activation of these systems will stimulate protein synthesis in the myocytes and fibroblasts, which will contribute to hypertrophy and fibrosis (Florea and Cohn, 2014; Sayer and Bhat, 2014; Azevedo *et al.*, 2016). Cardiac angiotensin II is an important *local* factor involved in cardiac remodeling following infarction (Sun, 2008).

CHAPTER 3
REPERFUSION INJURY

3.1. INTRODUCTION

As discussed previously, the impairment of blood flow to myocardial cells (ischemia) can result in tissue injury and organ dysfunction. The degree of the tissue damage will be determined by the duration and severity of the ischemic insult (Carden and Granger, 2000).

Reperfusion of ischemic tissue is viewed as the most effective strategy to salvage ischemic tissue, reduce infarct size and improve clinical outcome. The restoration of blood flow will replenish cellular ATP levels, re-establish ionic cell balance and restore organ function (Granger and Kvietys, 2015).

The options for reperfusion strategies following acute ST segment elevation myocardial infarction (STEMI) include: Primary percutaneous coronary intervention (PPCI), thrombolysis, rescue coronary angioplasty (R-PCI) and late PCI (>12 hours after symptoms) (Steg *et al.*, 2012).

In 1960 Jennings *et al*. made the observation that reperfusion per se however also contributes to myocardial injury. This was based on experiments using canine hearts subjected to coronary artery ligation in which reperfusion appeared to accelerate the development of necrosis. They observed that the histological changes following only 30-60 minutes of ischemia/reperfusion (I/R) equal the degree of necrosis normally observed after only 24 hours of ischemia (Jennings, 1960). Following this, reperfusion as the most successful treatment modality following ischemia was challenged by Reimer *et al*. (1977) in the 1970s. They also found that reperfusion can increase rather than reduce the extension of the infarct (Hearse, Humphrey and Chain, 1973; Reimer *et al.*, 1977). Hearse and colleagues (Hearse, Humphrey and Chain, 1973) then proposed that the reintroduction of oxygen to ischemic tissue results in an injury response not present during the period of hypoxia, and therefore not related to the ischemic event. This *reoxygenation-dependent injury response* is what is known today as “reperfusion injury” (Granger and Kvietys, 2015; Ferrari *et al.*, 2017). The evidence for reperfusion-dependent myocardial injury was summarized in 1985 by Braunwald

and Kloner (Braunwald and Kloner, 1985).

3.2. MANIFESTATIONS OF REPERFUSION INJURY

Myocardial reperfusion injury can manifest in different forms and includes (Neri *et al.*, 2017):

- Reperfusion induced arrhythmias, (Thandroyen *et al.*, 1988)
- Myocardial stunning, (Bolli, 1990)
- Micro-vascular obstruction,
- Lethal myocardial reperfusion injury, (Hausenloy and Yellon, 2013)
- Apoptosis, necrosis and
- Sudden death (Bonnemeier *et al.*, 2003).

3.2.1. Reperfusion induced arrhythmias

Reperfusion induced arrhythmias are commonly observed in patients undergoing reperfusion in the form of thrombolytic therapy or cardiac surgery. It is one of the causes for sudden death after the relief of coronary ischemia. Reperfusion arrhythmias may partly be attributed to the rapid and sudden alterations in ion concentrations. Gradual reperfusion therefore substantially decreases the frequency of malignant arrhythmias (Yamazaki *et al.*, 1986; Collard and Gelman, 2001).

Even though reperfusion may induce arrhythmias, research has shown a lower incidence of ventricular fibrillation or tachycardia in reperfused, than in persistent ischemic patients. This suggests that reperfusion lowers the *overall* risk of myocardial arrhythmias in the ischemic setting (Maxwell and Lip, 1997; Collard and Gelman, 2001).

3.2.2. Myocardial Stunning

Following reperfusion, mitochondrial oxidative phosphorylation returns to the (normal) pre-ischemic levels; contractile power, however, lags behind and only gradually normalizes (provided the ischemic period was not prolonged and resulted in infarction). This phenomenon of contractile dysfunction during reperfusion is termed myocardial stunning (Kloner and Jennings, 2001).

Myocardial stunning therefore refers to the reversible post-ischemic contractile

(myocardial) dysfunction that persists after reperfusion despite the absence of irreversible damage. This contractile dysfunction is fully reversible with time, although inotropic or mechanical circulatory support may be required (van der Horst *et al.*, 2003). Severe stunning may result in a low cardiac output and a decreased mechanical efficiency, since the stunned myocardium has a relatively excessive oxygen consumption for a certain amount of contractile work (Collard and Gelman, 2001; Turer and Hill, 2010).

Stunning is most likely caused by free radical mediated stress and intracellular calcium overload (Bolli, 1990). Other hypotheses include extracellular collagen alterations, myofilament malfunction, reduced sympathetic response, coronary microvascular spasm or plugging and a reduced ATP reserve (Bolli, 1990; Maxwell and Lip, 1997; Collard and Gelman, 2001).

3.2.3. Micro-vascular obstruction

In 1966 Krug *et al*. was the first to use the term microvascular obstruction (MVO). This refers to the observation that blood flow to an ischemic organ is often not fully restored after release of a vascular occlusion (Krug, De and Korb, 1966). In the setting of a STEMI, micro-vascular obstruction therefore describes suboptimal tissue perfusion despite restoration of flow to the ischemic area.

Major contributing factors to this ischemic-reperfusion associated “no reflow” phenomenon include (Maxwell and Lip, 1997; Ito, 2006; Luo and Wu, 2006; Heusch *et al.*, 2009; Kleinbongard *et al.*, 2011):

- Micro-embolization from the atherosclerotic plaque.
- Platelet micro-thrombi.
- The release of vasoconstrictor and thrombogenic substances.
- The release of inflammatory substances, leading to neutrophil plugging, increased leukocyte–endothelial cell adhesion and platelet–leukocyte aggregation.
- Damage to the capillary bed with impaired endothelium-dependent vasorelaxation.

- External capillary compression by swollen endothelial cells, swollen cardiomyocytes and interstitial fluid accumulation.

In 1983 Romson *et al*. highlighted the important role of leukocytes in micro-vascular obstruction, by demonstrating that leukocyte depletion improves coronary blood flow, decreases myocardial infarct size, and attenuates the incidence of ventricular arrhythmias (Romson *et al.*, 1983).

Microvascular obstruction contributes to reperfusion injury by being associated with a larger infarct size, myocardial stunning, lower left ventricle ejection fraction, adverse left ventricular remodeling, and worse clinical outcomes (Ito *et al.*, 1996; Wu *et al.*, 1998; Hausenloy and Yellon, 2013).

The general consensus is that microvascular obstruction is primarily related to reperfusion injury. Khan *et al.* (2014) however demonstrated that microvascular obstruction is present in all forms of reperfusion (early and late) *and* in patients that did not receive any form of reperfusion therapy. They therefore suggested that MVO is primarily related to ischemia, rather than reperfusion injury *per se* (Khan *et al.*, 2014).

3.2.4. Lethal myocardial reperfusion injury

Reperfusion-induced death of cells that were still viable at the end of an ischemic event is defined as lethal myocardial reperfusion injury (Piper, Garcña-Dorado and Ovize, 1998).

The major contributory factors include oxidative stress, calcium overload, mitochondrial permeability transition pore (MPTP) opening, and hyper-contracture (Yellon and Hausenloy, 2007). The existence of lethal myocardial reperfusion injury has been deduced from the observation that therapeutic interventions applied only at the onset of myocardial reperfusion reduced infarct size by 40%–50% (Yellon and Hausenloy, 2007). This suggests that lethal myocardial reperfusion injury may be responsible for up to 50% of the final myocardial infarct size (Hausenloy and Yellon, 2013).

Reperfusion associated injury response is however much wider than only cardiac manifestations. Other conditions where ischemia followed by reperfusion may form a

part of the pathophysiology include sickle cell disease, (Hebbel, 2014) osteoarthritis, (Vos *et al.*, 2012) obstructive sleep apnea, (Ryan, Taylor and McNicholas, 2005) Alzheimer's disease (Zheng *et al.*, 2010) and the fact that hepatic ischemic reperfusion promotes the metastasis of cancerous cells to the liver from the pancreas (Lenglet, Mach and Montecucco, 2012; Yoshimoto *et al.*, 2012) and colorectum (Lenglet, Mach and Montecucco, 2012; Granger and Kvietys, 2015).

3.3. PATHOPHYSIOLOGY OF REPERFUSION INJURY

Several reperfusion-associated theories have been proposed to explain why reperfusion itself is known as a "double-edged sword" (Braunwald and Kloner, 1985).

The exact mechanisms of reperfusion injury are not fully known, (Baines, 2011) but it could be due to the confluence of multiple pathways: electrolyte and pH alterations, cytosolic and mitochondrial calcium overload, release of reactive oxygen species, opening of the mitochondrial permeability pore, inflammation, endothelial dysfunction, autophagy and alterations in the Calpain system.

All above mentioned factors play a role in producing reversible and irreversible damage to ischemic reperfused cardiomyocytes (Neri *et al.*, 2017).

3.3.1. Electrolyte and pH alterations

During ischemia, accumulation of intracellular hydrogen ions (H^+) (causing cellular acidosis) as a consequence of anaerobic glycolysis. During reperfusion sodium (Na^+) dependent pH regulatory mechanisms (Na^+-H^+ exchanger and the sodium - bicarbonate (HCO_3^-) transporter) are activated, which lead to intracellular sodium accumulation and normalization of the pH (Tani and Neely, 1989). This rapid normalization of pH contributes to cytotoxicity, (Lemasters *et al.*, 1996) and the increase in intracellular sodium activates the sarcolemmal $2Na^+$- Calcium (Ca^{2+}) exchanger, resulting in an exchange of intracellular sodium for extracellular calcium. This leads to intracellular calcium overload and cell death (Yellon and Hausenloy, 2007; Frank *et al.*, 2012). An increase in calcium entry via sarcolemmal L-type calcium channels and a deficient uptake of cytosolic calcium into the sarcoplasmic reticulum by Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) further contribute to this calcium overload (Turer and Hill, 2010).

Calcium overload is detrimental because, during reperfusion, the mitochondria may use the newly restored oxygen for calcium transport instead of for ATP production, two processes which compete for the same energy source (Ferrari and Williams, 1986; Ferrari *et al.*, 2017). Other consequences include myofibril hyper contractility, ultrastructural damage to mitochondria, and myocardial stunning (Nayler, 1981; Turer and Hill, 2010; Ferrari *et al.*, 2017).

3.3.2. Reactive oxygen species (ROS)/ oxidative stress

Reactive oxygen species (ROS, previously known as oxygen-derived free radicals) are a well-established contributing factor to reperfusion injury (Granger and Kvietys, 2015). Oxidative stress occurs as a result of the reintroduction of oxygen after a period of ischemia in combination with the incomplete reduction of reactive oxygen species during the reperfusion period. During reperfusion of ischemic tissue an imbalance therefore exists between the generation rate of reactive oxygen species (superoxide, hydroxyl radical, peroxynitrite and hydrogen peroxide) and the tissue's ability to clear these reactive species (Granger and Kvietys, 2015; Ferrari *et al.*, 2017).

A major contributory factor to the incomplete reduction of ROS is the fact that ischemia results in a reduction of the natural scavengers against oxygen free radicals. This includes a decrease in *superoxide dismutase* (SOD), *catalase*, *glutathione-peroxidase* and the *thioredoxin reductase* systems. Ischemia also causes a washout of sarcolemmal *α-tocopherol* (an anti-oxidant) (Ferrari *et al.*, 1992; Curello *et al.*, 1995). (Refer to Figure 3.1)

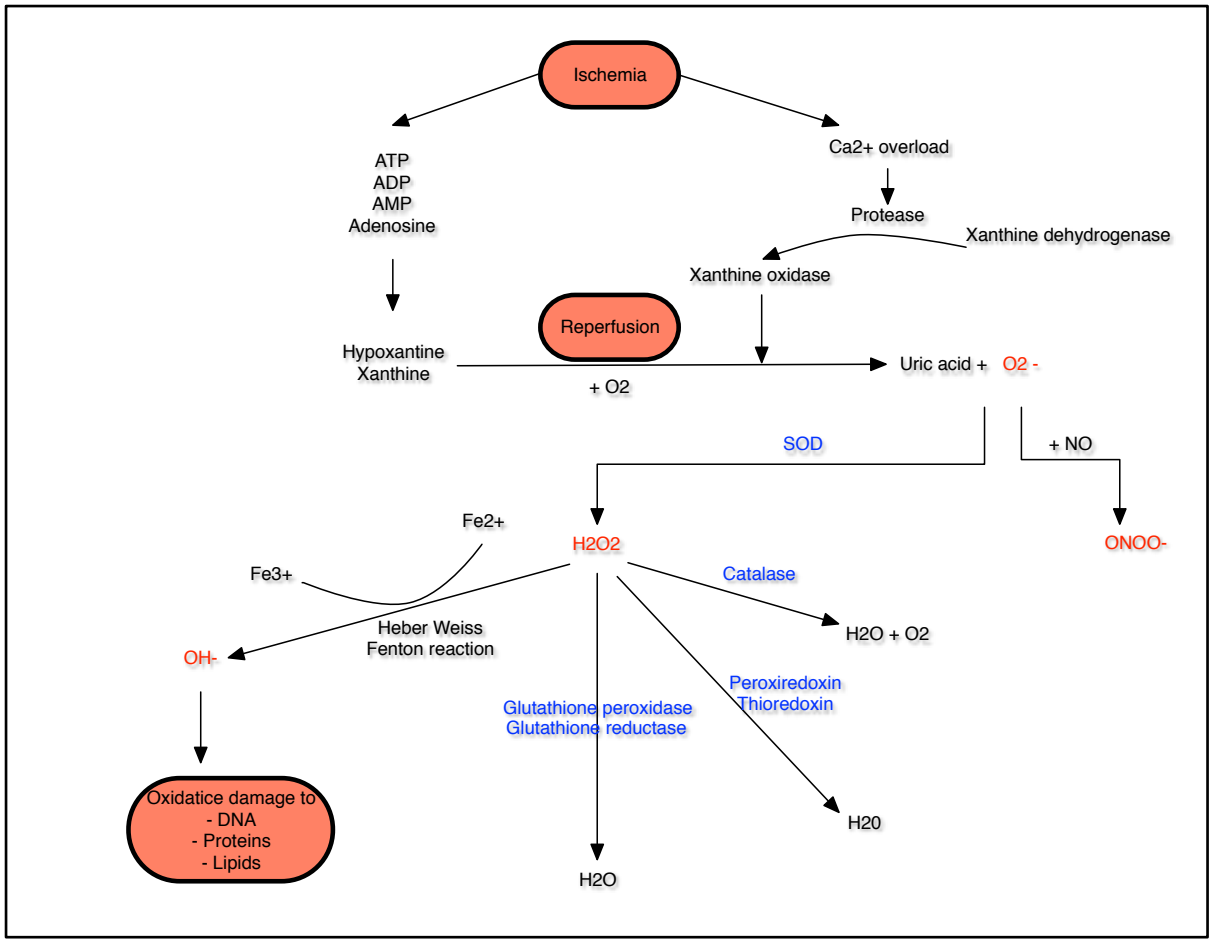


Figure 3.1: Reactive oxygen species production as a result of ischemia and reperfusion

Superoxide (O_2-) and hydrogen peroxide (H_2O_2) are generated invariably in normal cells, and serve as precursors for highly reactive oxygen intermediates. H_2O_2 is reduced to H_2O by either catalase, (Goodsell *et al*., 2015) peroxiredoxin or glutathione peroxidase. Glutathione reductase reduces the oxidized form of the enzyme glutathione peroxidase (Deponte 2013). The Haber–Weiss reaction generates •OH (hydroxyl radicals) from H_2O_2 and $\bullet O_2^-$. The first step of the catalytic cycle involves reduction of ferric ion to ferrous: $Fe^{3+} + \bullet O_2^- \rightarrow Fe^{2+} + O_2$. The second step is the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$. Net reaction: $\bullet O_2^{--} + H_2O_2 \rightarrow \bullet OH + OH^- + O_2$ (Koppenol, 2001).

Potential **sources of ROS** secondary to ischemia and reperfusion include xanthine oxidase, NADPH oxidase, mitochondria, nitric oxide synthase, cytochrome P450, lipoxygenase/ cyclooxygenase and monoamine oxidase (Granger and Kvietys, 2015).

3.3.2.1. Xanthine dehydrogenase and Xanthine oxidase

Xanthine dehydrogenase (XDH) and xanthine oxidase (XO) control the rate-limiting step of purine catabolism i.e. the hydroxylation of xanthine to uric acid. XDH uses NAD^+ as an electron acceptor and XO uses O_2 as the terminal electron acceptor, thereby having the ability to generate reactive oxygen species (Thompson-Gorman and Zweier, 1990; Granger and Kvietys, 2015).

During ischemia hypoxanthine, xanthine and succinate accumulate (Chouchani *et al.*, 2014) and XDH is converted to XO (this is mediated by limited proteolysis and/or sulfhydryl oxidation). The re-introduced oxygen (during reperfusion) in the presence of the accumulated xanthine then reacts with XO to produce a burst of superoxide (Granger and Kvietys, 2015).

Xanthine oxidase activity is reliant on oxygen tension (as evidenced by studies demonstrating that an inverse relationship exists between O_2 tension and XO activity) (Poss *et al.*, 1996), a variety of cytokines (IL-1, IFN-γ, IL-6 and TNF-α) and inflammatory mediators (Pfeffer, Hueckstead and Hoidal, 1994; Granger and Kvietys, 2015).

Xanthine oxidase has also been implicated in the leukocyte and neutrophil recruitment that occurs following ischemic reperfusion injury (Zimmerman, Grisham and Granger, 1990; Granger and Kvietys, 2015). It therefore has been proposed that the inflammatory role of XO-derived ROS may play a more important role than XO-derived ROS directly mediating tissue injury (Granger, 1988; Meneshian and Bulkley, 2002).

3.3.2.2. NADPH oxidase as source of ROS

The Nox/Duox family of NADPH oxidases also plays an important role in the production of ROS following ischemia and reperfusion. This family of multiprotein complexes comprise of 7 members: Nox-1 to Nox-5 and the dual oxidases (Duox)-1 and -2. The Duox enzymes predominately produce hydrogen peroxide along with Nox-4, while the remaining Nox iso-enzymes are responsible for superoxide production (Kleikers *et al.*, 2012).

Mice studies, using the buffered perfused Langendorff model, showed that the Nox-1 and 2 isoform deletions play an important role in myocardial protection against reperfusion injury (Braunersreuther *et al.*, 2013).

All Nox homologs (except Nox-4) are constitutively inactive and require cell stimulation to generate ROS. Following NADPH activation, the Nox enzymes can generate superoxide via one-electron reduction of molecular oxygen using cytoplasmic NADPH as an electron donor (Figure 3.2) (Pendyala *et al.*, 2009; Granger and Kvietys, 2015).

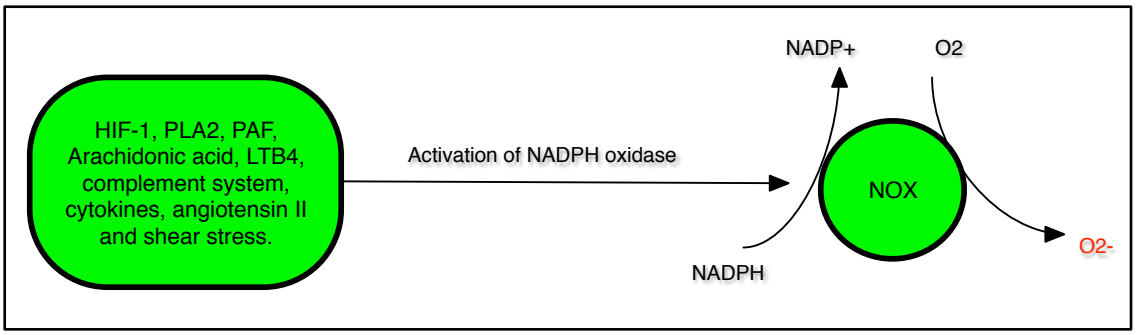


Figure 3.2: NADPH as a source of superoxide

NADPH, NOX enzyme dependent superoxide generation. Abbreviations: NADPH: nicotinamine adenine dinucleotide phosphate oxidase, HIF: hypoxia inducible factor, PLA: phospholipase, PAF: platelet-activating factor, LT: leukotriene.

Hypoxia that accompanies ischemia, prompts the production and release of hypoxia inducible factor-1α (HIF-1α), which in turn promotes the production and activation of Nox (Rupin *et al.*, 2004). This also activates a positive feedback loop wherein Nox-derived reactive oxygen species stimulate the production of HIF-1α (Kleikers *et al.*, 2012).

During ischemia and at the time of reperfusion, different chemical mediators are released with the potential to activate NADPH oxidase. This includes phospholipase A2(PLA2), platelet-activating factor (PAF), arachidonic acid and its metabolic products thromboxane and leukotriene (e.g., LTB4) (Dewald and Baggiolini, 1985; Granger and Kvietys, 2015). Other factors responsible for an increase in NADPH activity secondary to I/R include the activation of the complement system, (Arumugam *et al.*, 2004) cytokines (e.g., TNF-α, IL-1β), (Barth, Stewart-Smeets and Kuhn, 2009) angiotensin II (the best characterized stimulant for NADPH oxidase) (Cat *et al.*, 2013) and the reduction in shear stress that occurs during ischemia. This reduction results in endothelial cell membrane depolarization via inactivation of ATP-sensitive potassium channels, which leads to activation of NADPH oxidase (Granger and Kvietys, 2015).

3.3.2.3. Mitochondria as a ROS source

Mitochondria (although essential for energy supply to the cardiomyocytes via mitochondrial oxidative phosphorylation (OXPHOS)) are another major source of reactive oxygen species (Kalogeris, Bao and Korthuis, 2014; Zorov, Juhaszova and Sollott, 2014).

The movement of electrons through the electron transport chain (ETC) can result in leakage of electrons, thereby generating reactive oxygen species during the course of normal oxidative phosphorylation (Zorov, Juhaszova and Sollott, 2014). The electron transport chain consists of multi-subunit complexes (complexes I–IV) in the inner mitochondrial membrane which are coupled to mobile carriers (coenzyme Q and cytochrome c) (Murphy, 2009; Zorov, Juhaszova and Sollott, 2014). All of these components have redox potential making it possible for them to transfer single electrons to O_2 to form O_2^- (Murphy, 2009; Lenaz, 2012).

The mitochondrial electron transport chain is therefore considered to be the major source of ROS during normal oxidative metabolism (Murphy, 2009; Kalogeris, Bao and Korthuis, 2014; Zorov, Juhaszova and Sollott, 2014). The normally low level of detectible ROS can be attributed to the presence of an extensive intra-mitochondrial antioxidant system (consisting of superoxide dismutase, catalase, glutathione peroxidase and peroxiredoxin) (Kowaltowski *et al.*, 2009; Dröse and Brandt, 2012). The net production of ROS by mitochondria is therefore determined by the balance between the generation and the scavenging of ROS (Kowaltowski *et al.*, 2009).

Oxidative stress results in the peroxidation of cardiolipin which plays a critical part in electron transport chain complex assembly, stability and electron transfer. With cardiolipin ineffective, complexes can disassemble with additional generation of reactive oxygen species (Paradies *et al.*, 2004; Petrosillo *et al.*, 2005).

Other mitochondrial sources of reactive oxygen species include monoamine oxidase, (Kaludercic *et al.*, 2014) succinate, (Hamel *et al.*, 2014) pyruvate dehydrogenase (PDH), α- ketoglutarate dehydrogenase (α-KDH) complexes, (Starkov, 2013) α-glycerophosphate dehydrogenase, (Adam-Vizi and Tretter, 2013) and aconitase (an enzyme located in the mitochondrial matrix) (Granger and Kvietys, 2015).

3.3.2.4. Nitric oxide synthase (NOS) as source of reactive oxygen species

Oxidative stress is associated with the production of reactive oxygen species as well as reactive *nitrogen* species (RNS) (Navarro-Yepes *et al.*, 2014). The production of nitric oxide (NO) is considered another key factor in reperfusion injury. Nitric oxide has been implicated in a cardioprotective as well as a cardiotoxic role (Figure 3.3).

Nitric oxide minimizes ROS-mediated damage by means of the following (Moncada and Higgs, 1993; Jones and Bolli, 2006; Neri *et al.*, 2017):

1. Acting as a free radical scavenger.
2. Promotion of vasodilation.
3. Inhibition of platelet aggregation and adhesion.
4. Inhibition of neutrophil adhesion and inflammation.

Nitric oxide excess can however also induce cellular injury either due to direct toxicity or secondary to the formation of the cytotoxicity oxidant peroxynitrite ($ONOO_2$) following a reaction with superoxide (O_2^-) (Turer and Hill, 2010; Neri *et al.*, 2017).

During reperfusion an increase in nitric oxide release may be the result of shear stress in the coronary vasculature, increased intracellular calcium and a combination of L-arginine and molecular oxygen (Zweier, Fertmann and Wei, 2001). Nitric oxide is produced endogenously within the myocardium by three distinct isoforms of NO synthase (NOS). Neuronal NOS (NOS1) and endothelial NOS (NOS3), which are always present within the cardiomyocytes, while inducible NOS (NOS2) is only expressed during an inflammatory response (Neri *et al.*, 2017).

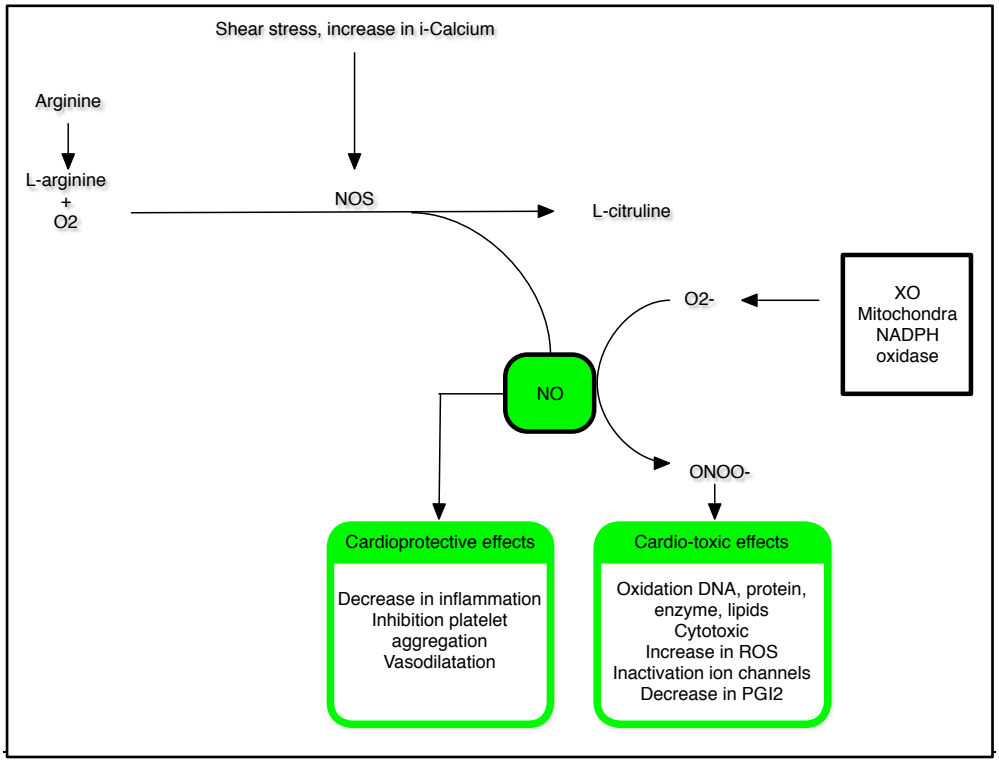


Figure 3.3: The role of Nitric oxide as a source of reactive oxygen species during reperfusion

Abbreviations: O2: oxygen, NO: nitric oxide, NOS: NO synthase, XO: xanthine oxidase, ROS: reactive oxygen species, PGI2: prostacyclin, ONOO-: peroxynitrite, NADPH: nicotinamide adenine dinucleotide phosphate oxidase.

The consequences of an increase in reactive oxygen species can be summarised as follows:

- Direct damage to cellular nucleic acids (DNA), proteins and lipids (Cain *et al.*, 1999).
- An increased susceptibility to proteolysis in cardiomyocytes (Neri *et al.*, 2017).
- Activation of pro-inflammatory pathways (stress response) resulting in tumor necrosis factor alpha (TNFa) production (Cain *et al.*, 1999; Frank *et al.*, 2012).
- Opening of the mitochondrial Permeability Transition Pore (mPTP), (Zorov *et al.*, 2009) resulting in a positive feedback loop of additional free radical release from the mitochondria ("ROS-induced ROS release") (Zorov *et al.*, 2000; Turer and Hill, 2010).
- All of the above, in combination with the activation of metalloproteinases and calpains contribute to swelling and lysis of cells, which can result in cell dysfunction and death (Neri *et al.*, 2017).

3.3.3. Mitochondrial Permeability Transition Pore (mPTP)

Cardiac myocytes use large amounts of energy, and therefore have a high density of mitochondria. As part of this, the **mitochondrial Permeability Transition Pore** (mPTP), a nonselective channel of the inner mitochondrial membrane, plays a critical role in reperfusion injury (Heusch, Boengler and Schulz, 2010; Turer and Hill, 2010).

The mPTP, responsible for maintaining mitochondrial transmembrane potential, is closed under physiological conditions (He and Lemasters, 2002) and therefore impermeable to ions and proteins. During myocardial ischemia, the susceptibility of the mPTP is increased but it remains closed secondary to the low pH (Griffiths and Halestrap, 1995) .

Triggers for the opening of the mPTP include:

- Mitochondrial calcium overload (Orrenius, Zhivotovsky and Nicotera, 2003).

- Restoration of a physiological pH (Kim, Jin and Lemasters, 2006).
- Increased levels of phosphate (Halestrap, Clarke and Javadov, 2004).
- ATP depletion (Halestrap and Pasdois, 2009).
- Oxidative stress (Kim, Jin and Lemasters, 2006; Turer and Hill, 2010).

These triggers collectively contribute to closure of the mPTP during ischemia and the opening thereof during the first few (2-3) minutes of reperfusion, as first demonstrated by Griffiths and Halestrap (Griffiths and Halestrap, 1995). This was subsequently confirmed by others (Ong *et al.*, 2015; Neri *et al.*, 2017).

Opening of this pore creates a non-selective channel between the inner membrane of the mitochondrion and the sarcoplasm, allowing passage through the inner mitochondrial membrane of all molecules <1.5 kDa (Ferrari *et al.*, 2017).

This results in a major problem for the still viable cells since opening of the channel causes a loss of the electrochemical gradient, it collapses the mitochondrial membrane potential and uncouple oxidative phosphorylation leading to the impairment of ATP generation and cell necrosis (Griffiths and Halestrap, 1995; Frank *et al.*, 2012; Neri *et al.*, 2017). The opening of the mPTP also causes mitochondrial swelling, with potential outer mitochondrial membrane rupture, favoring the deposition of proapoptotic factors. This, in combination the increase in calcium flow into the mitochondria (which triggers different caspases), will initiate apoptotic cell death (Ong *et al.*, 2015; Ferrari *et al.*, 2017). Opening of the mPTP also causes loss of mitochondrial cytochrome C, leading to apoptosis (Wigdal *et al.*, 2004).

According to Frank *et al*. (2012), the fate of the cell during reperfusion is determined by the extent of mitochondrial membrane permeability. The cell may either recover, undergo programmed cell death or die from necrosis (due to inadequate energy production) (Frank *et al.*, 2012). This is a major contributing factor to cell death post coronary perfusion in cells that survived the initial ischemic insult (Morciano *et al.*, 2015; Ferrari *et al.*, 2017).

3.3.4. Role of inflammation

Endothelial activation and injury secondary to ischemic reperfusion increase vascular permeability and result in recruitment of inflammatory cells.

Excessive TNF alpha expression (secondary to reactive oxygen intermediates) causes direct damage to cellular components and also activates the stress response. The subsequent cardiomyocyte TNF receptor type 1-stimulation results in contractile dysfunction, hypertrophy, fibrosis and cell death (Kleinbongard, Schulz and Heusch, 2011).

The increase in the intracellular calcium concentration in combination with the generation of calcium pyrophosphate complexes and the formation of uric acid is also a potent stimulator of inflammation. It can bind to intracellular protein complexes called inflammasomes, (Kawaguchi *et al.*, 2011) resulting in the production and secretion of interleukin-1 (IL-1) β. The calcium complexes in combination with uric acid also stimulate Toll-like receptors, (Chao, 2009) leading to the secretion of pro-inflammatory cytokines and chemokines through the activation of NF-κB (Arslan, Kleijn and Pasterkamp, 2011; Frank *et al.*, 2012).

During the first 6 hours of myocardial reperfusion, the release of chemo attractants draws neutrophils into the infarct zone and during the next 24 hours they migrate into the myocardial tissue. Cell-adhesion molecules facilitate this process via releasing intercellular ICAM-1, VCAM-1 and E-selectin (in response to the injured endothelium) which promote tissue invasion by inflammatory cells. The neutrophils are responsible for microvascular occlusion, the release of degrading enzymes (proteases) and generation of reactive oxygen species (Yellon and Hausenloy, 2007; Turer and Hill, 2010; Frank *et al.*, 2012).

3.3.5. Other mechanisms of reperfusion injury

Proteolysis within the cardiac sarcomere is regulated by the ubiquitin proteasome, autophagy and the calpain systems (Portbury, Willis and Patterson, 2011). An imbalance in proteolysis will lead to sarcomeric dysfunction which is associated with hypoxia, reperfusion injury, myocardial infarction, and end-stage heart failure.

The key role players in the activation of the **calpain system** are the increase in intracellular calcium levels as well as the normalization of intracellular pH during reperfusion. Uncontrolled calpain activation has been implicated in the pathophysiology of myocardial reperfusion injury (Khalil *et al.*, 2005; Neri *et al.*, 2017).

Autophagy is another contributing factor implicated in the pathogenesis of reperfusion injury, (Matsui *et al.*, 2007) and will be discussed in Chapter 4.

3.4. CONTROVERSIES SURROUNDING THE EXISTENCE OF MYOCARDIAL REPERFUSION INJURY

Reperfusion injury has been a subject of debate for the past three decades, the main dispute being the contributory factors to tissue injury. Is myocardial injury only a result of the ischemic period, or does reperfusion extends tissue injury due to the mechanisms discussed in Section 3.3 (Pathophysiology of reperfusion injury)? The majority of questions and queries are aimed at one specific manifestation of reperfusion injury, *lethal myocardial reperfusion injury* (Section 3.2.4). As discussed earlier, it specifically refers to cell death, following ischemia, which can be prevented by interventions applied at the time of reperfusion (Piper, Dorado and Ovize, 1998). It therefore implies that a part of cell death, following ischemic reperfusion injury, occurs as a consequence of reperfusion *per se* (Garcia-Dorado, Ruiz-Meana and Piper, 2009).

A significant contribution to cell death as a result of lethal reperfusion injury is supported by some investigators (Jolly *et al.*, 1984; Ambrosio *et al.*, 1986; Piper, Garcña-Dorado and Ovize, 1998; Hausenloy and Yellon, 2013), while others (Uraizee *et al.*, 1987; Richard *et al.*, 1988; Nejima *et al.*, 1989; Ganz *et al.*, 1990; Ytrehus *et al.*, 1994) disregard the role it plays as a contributing factor to myocyte death during reperfusion.

It has been difficult to prove the existence of such a phenomenon, because reperfusion injury cannot be studied in isolation (without pre-existing ischemia) (Kloner, 1993). The existing evidence and current knowledge, with regard to reperfusion injury, are therefore based on the (myocardial protective) result of therapeutic interventions applied at the start of reperfusion (Yellon and Hausenloy, 2007). The rationale for these studies is that “if an agent given at the time of reperfusion reduces myocardial infarct size to a greater extent than does reperfusion alone, then some cell death must have recurred at the time of reperfusion”. Furthermore, the agent should have acted by reducing lethal reperfusion injury, because it was given during the phase of reperfusion only (Kloner, 1993).

Ganz *et al*. (1990), using an eloquent experimental model in which one part of the ischemic bed was reperfused while the other part was not reperfused, failed to demonstrate that reperfusion contributed to lethal myocyte injury (Ganz *et al.*, 1990).

Other studies employing the pharmacological modification of reperfusion injury, by administration of the drug at the time of reperfusion, however support lethal myocardial reperfusion injury. It includes studies done with:

- Oxygen free radical scavengers (Jolly *et al.*, 1984; Kloner, Przyklenk and Whittaker, 1989; Horwitz *et al.*, 1994; Dhalla *et al.*, 2000).
- Calcium channel blockers (Théroux *et al.*, 1998).
- Adenosine (Ely and Berne, 1992; Vinten-Johansen *et al.*, 1999) and
- Opioid receptor ligands (Gross and Gross, 2006).
- Prolonging intracellular acidosis at the onset of reperfusion (Inserte *et al.*, 2009).

The myocardial protective effect as a result of postconditioning (Kin *et al.*, 2004; Vinten-Johansen *et al.*, 2005; Zhao and Vinten-Johansen, 2006), reperfusion induced therapeutic hypothermia (Miki *et al.*, 1998; Bernard *et al.*, 2002) as well as the discovery of the reperfusion injury salvage kinase (RISK) pathway, (Hausenloy and Yellon, 2004) provide further support for the contribution of lethal myocardial reperfusion injury to myocyte death.

Possible explanations for the discrepancies in results include a variation in ischemic and reperfusion duration, administration of drugs during both ischemic and reperfusion phases, different experimental models, no standardization in terms of coronary collateral flow within the ischemic bed, and uncertainty as to the dose and duration of drug administration (Kloner, 1993).

The confirmation that lethal reperfusion injury contributes significantly to cell death following myocardial ischemia, is thus based on discoveries in the field of cardioprotection with different drugs (applied at the time of reperfusion) and postconditioning (Gross and Auchampach, 2007; Rodríguez-Sinovas *et al.*, 2007; Garcia-Dorado, Ruiz-Meana and Piper, 2009).

3.5. THERAPEUTIC STRATEGIES FOR REDUCING MYOCARDIAL REPERFUSION INJURY

Cardiac ischemia is either unpredictable (myocardial infarction) or inevitable (in the operating room) – and the best therapeutic strategy against ischemic damage is reperfusion. The problem, as mentioned before, is the tissue damage following reperfusion. There is currently no therapeutic agent available to completely prevent microvascular obstruction and lethal myocardial reperfusion injury following reperfusion (Hausenloy and Yellon, 2013). Thus, there is a great deal of interest in therapeutic options that will minimize reperfusion-mediated injury (Turer and Hill, 2010).

Therapeutic targets include ischemic conditioning, hyperoxia, hypothermia, oxidative stress, calcium overload, pH correction and inflammation (Hausenloy and Yellon, 2013).

3.5.1. Ischemic conditioning

Ischemic conditioning includes ischemic preconditioning, remote ischemic pre-conditioning and ischemic post-conditioning. These three endogenous strategies have the potential to protect the heart from the detrimental effects of reperfusion injury as will be described below.

Ischemic preconditioning

In 1986, Murry and Reimer described a process whereby repetitive brief ischemic episodes, preceding a prolonged period of ischemia with reperfusion, resulted in a significantly reduced infarct size in dogs (the so-called ischemic preconditioning) (Murry, Jennings and Reimer, 1986). Ischemic preconditioning is the second most (reperfusion is first) powerful intervention to reduce infarct size in ischemic hearts. Almost 9000 papers have been published on this topic in the last 30 years (Hausenloy and Yellon, 2016).

The *human* heart can also be preconditioned: it was demonstrated that ischemic preconditioning has a protective effect on recovery of ventricular contractility in patients post coronary artery bypass grafting (Wu *et al.*, 2000). A meta-analysis including 22 trials and a total of 933 patients, found that ischemic preconditioning

reduces ventricular arrhythmias, decrease inotropic requirements, and shortened the length of stay in an intensive care unit (Walsh *et al.*, 2008; Hausenloy and Yellon, 2016).

The need to intervene directly on the heart, the inherent risk of thrombo-embolization and the requirement to intervene *before* the ischemic event; have however prevented ischemic preconditioning from being adopted in the clinical setting (Hausenloy and Yellon, 2016).

Ischemic postconditioning

Ischemic postconditioning refers to gradual or interrupted reperfusion, at the onset of reperfusion, using short (second) episodes of ischemia/reperfusion, leading to myocardial protection.

This intervention has been shown to reduce myocardial infarct size in animals and humans, although the cardioprotective effects of ischemic postconditioning do not seem to be as robust as ischemic preconditioning (Tsang, Hausenloy and Yellon, 2005; Hausenloy and Yellon, 2016). In addition, the results from studies investigating ischemic postconditioning are controversial: beneficial, (Ma *et al.*, 2006; Favaretto *et al.*, 2014; Touboul *et al.*, 2015) no benefit, (Sorensson *et al.*, 2010; Hahn *et al.*, 2013; Khalili *et al.*, 2014; Engstrøm *et al.*, 2017) and even detrimental (Freixa *et al.*, 2011; Tarantini *et al.*, 2012) effects have been reported.

Of these, the second largest clinical study, which included 700 patients, showed no beneficial effect of ischemic postconditioning in terms of myocardial protection (Hahn *et al.*, 2013). The most recent and largest study is a Danish multicenter, randomized clinical trial that included 1234 patients. It was concluded that routine ischemic postconditioning does not improve clinical outcome in patients with ST-segment elevation myocardial infarction (Engstrøm *et al.*, 2017).

Remote ischemic conditioning

The heart can also be protected against ischemic reperfusion injury from a distance i.e. by applying one or more cycles of brief ischemia and reperfusion to another organ or tissue. This phenomenon has been termed remote ischemic preconditioning (RIPC) (Przyklenk *et al.*, 1993).

Remote ischemic preconditioning has been reported to show organ protection in patients undergoing cardiovascular surgery, (Cheung *et al.*, 2006; Ali *et al.*, 2007; Hausenloy *et al.*, 2007; Thielmann *et al.*, 2010; Heusch *et al.*, 2012) who had a coronary intervention (Hoole *et al.*, 2009; Bøtker *et al.*, 2010) or a stroke (Hougaard *et al.*, 2014). However the results were variable: some of these studies reported improved clinical outcomes, (Hoole *et al.*, 2009; Bøtker *et al.*, 2010) while other trials failed to show beneficial effects (Rahman *et al.*, 2010; Lucchinetti *et al.*, 2012; Meybohm *et al.*, 2013). Possible explanations for the contradictory results include the fact that the studies differ with regard to their end points, samples sizes, study design, anesthesia regimen and also whether it was a single or multicenter study (Meybohm *et al.*, 2015). Individual patient comorbidities (diabetes) and chronic drugs (sulfonylureas, ant-platelet therapy, glyceryl nitrate) will also play a major role in the lack of consistency in terms of outcome, as well as translation of results, in terms of remote ischemic preconditioning.

The large-scale (1403 patients), multicenter, double-blind, randomised trial done by Meybohm *et al*. (2015) showed that there was no significant difference between upper-limb remote ischemic preconditioning and a sham intervention with respect to the rate of postoperative myocardial infarction, stroke, renal failure, and 90-day mortality post elective cardiac surgery (Hausenloy *et al.*, 2015; Meybohm *et al.*, 2015). These neutral results are consistent with those of a meta-analysis done in 2014 wherein the authors included 23 trials of remote ischemic preconditioning (2200 patients). It was found that RIPC did not have a significant effect on clinical end points, death, myocardial infarction, acute renal failure, stroke, mesenteric ischemia, and hospital or critical care length of stay (Trialists' Group *et al.*, 2014). A 2017 Cochrane review of the effect of RIPC in patients undergoing coronary artery bypass graft surgery (with or without valve surgery) supports these findings. This review concluded that there is no evidence of RIPC having a treatment effect on clinical outcomes (which included all-cause mortality, non-fatal myocardial infarction or any new stroke, or both, assessed at 30 days after surgery) (Benstoem *et al.*, 2017).

3.5.2. Therapeutic hyperoxemia and hypothermia

Myocardial protective effects secondary to therapeutic hypothermia can be attributed to reducing the metabolic demand and the inflammatory response, stabilization of mitochondrial permeability, production of nitric oxide, equilibration of reactive oxygen species, calcium channels homeostasis, decreasing platelet aggregation and increasing myocardial efficiency (Carden and Granger, 2000; Chavez *et al.*, 2017).

Animal studies have established that therapeutic hypothermia may decrease infarct size (Dae *et al.*, 2002; Tissier *et al.*, 2012) or have a cardioprotective effect (Dae *et al.*, 2002; X. Yang *et al.*, 2011). The studies done in humans however yielded controversial results (Chavez *et al.*, 2017).

Initially both the COOL-MI and ICE-IT human trials, aimed to assess the effect of mild therapeutic hypothermia on infarct size in STEMI patients, displayed disappointing results. A later data review revealed that patients recruited to these trials had undergone prolonged initial ischemia without induction of therapeutic hypothermia. The adjusted results, following re-analysing the data, demonstrated a reduction in infarct size in anterior myocardial infarction (Erlinge *et al.*, 2013; Chavez *et al.*, 2017). The RAPID-MI ICE trial, using therapeutic hypothermia as an adjunctive therapy with PCI in patients with STEMI, demonstrated a significant infarct size reduction (Gotberg *et al.*, 2010). A follow-up multicenter study, the CHILL-MI trial (n = 120) however could not reproduce the reduction in infarct size in the therapeutic hypothermia group (Erlinge *et al.*, 2014). The recent randomized SHOCK-COOL trail, in which mild hypothermia was used in patients with cardiogenic shock after acute myocardial infarction, also failed to show a substantial benefit (Fuernau *et al.*, 2018).

The unavoidable inconsistencies in human trials (infarct size and duration, heart failure, other co-morbid diseases and medication), as well as the timing of induction therapeutic hypothermia, are potentially the biggest contributory factors to the above mentioned conflicting results (Chavez *et al.*, 2017).

The reasoning for hyperbaric oxygen therapy is based on the following: When an ischemic insult deprives tissue of oxygen, the restoration of oxygenation through the microcirculation may stop or even reverse tissue necrosis. On the other hand, a

hyperoxic tissue environment following ischemia and reperfusion injury may increase ROS production and thus increase the degree of myocyte death resulting from reperfusion injury (Francis and Baynosa, 2017).

Hyperbaric oxygen therapy however appears to produce its beneficial effects on ischemic tissue by decreasing ROS production as well as increasing their degradation, inhibition of apoptosis following renal reperfusion injury, (Q. Zhang *et al.*, 2008; Migita *et al.*, 2016) decreasing the inflammatory response, (Q. Zhang *et al.*, 2008) increasing antioxidant enzyme expression, (Zhang and Gould, 2014) and reducing ICAM-1 expression and neutrophil adhesion (Buras *et al.*, 2000). There is also evidence that preconditioning, as a result of hyperbaric oxygen therapy, results in improved survival and microcirculatory perfusion (Xiao *et al.*, 2015).

Hyperbaric oxygen is therefore, from a theoretical point of view, supposed to reduce infarct size; this will be as a result of decreasing tissue edema, reducing formation of lipid peroxide radicals, altering nitric oxide synthase expression and inhibiting leukocyte adherence and microcirculatory plugging (Hausenloy and Yellon, 2013). Pooled data from AMIHOT I and II (Acute Myocardial Infarction With Hyperoxemic Therapy II) support this: It was found that in patients with anterior ST-segment elevation myocardial infarction, undergoing PCI within 6 hours of symptom onset, infusion of 90-minute intracoronary supersaturated oxygen in the left anterior descending artery infarct territory results in a significant reduction in infarct size (Stone *et al.*, 2009).

Extensive evidence from pre-clinical experiments and clinical studies in other patient groups however suggests more harm than good, this results from oxidative stress, vasoconstriction, perfusion heterogeneity and myocardial injury secondary to hyperoxia (Spoelstra-de Man *et al.*, 2015). Hyperoxia during cardiopulmonary bypass has also been reported to be potentially harmful and should be avoided unless it is used as a preventative treatment strategy in the setting of significant gas micro-emboli (Young, 2012).

3.5.3. Anti-inflammatory strategies

Key role players in the inflammatory response following ischemic-reperfusion injury

include the production of reactive oxygen species, complement activation, neutrophil activation and endothelial dysfunction (Dirksen *et al.*, 2007).

Therapeutic strategies to limit leukocyte-mediated reperfusion injury are summarized in the table below. These strategies have proven to be effective in ischemic *anima*l models (Panés, Perry and Granger, 1999).

Table 3.1: Therapeutic strategies to limit leukocyte-mediated reperfusion injury

Therapeutic area	**Relevant drug**
1. Inhibition of inflammatory mediator release or receptor engagement.	Soluble interleukin-1 receptor antagonists, anti–tumor necrosis factor alpha antibodies, platelet activation factor–leukotriene B_4 antagonists and biostable aspirin-triggered lipoxin analogs (Chiang *et al.*, 1999; Panés, Perry and Granger, 1999).
2. Leukocyte adhesion molecule synthesis.	Glucocorticoids, aspirin, salicylates, gold salts, D-penicillamine, anti- sense oligodeoxynucleotides and transcription factor decoys.
3. Leukocyte - endothelial adhesion.	Monoclonal antibodies.

Clinical trials using anti-inflammatory strategies (like polymorphonuclear cell (PMN)

inhibition) have however generally yielded disappointing results (Faxon *et al.*, 2002; Dirksen *et al.*, 2004).

3.5.4. Antioxidant therapy

Limited amounts of reactive oxygen species might result in cardiac protection via preconditioning (Tang *et al.*, 2002) while excessive production of ROS (as seen during reperfusion) is damaging to the myocardium (Neri *et al.*, 2017).

Animal studies have demonstrated the efficacy of antioxidant therapy in the prevention and attenuation of ischemic reperfusion injury. These include the use of superoxide dismutase, mannitol, allopurinol, iron chelating compounds, vitamin E, *N*-acetylcysteine, catalase, angiotensin-converting enzyme inhibitors and calcium channel antagonists (Maxwell and Lip, 1997). Two human studies also demonstrated the beneficial effects of superoxide dismutase (Marzi *et al.*, 1993; Land, 1994). However, in the majority of human studies, the efficacy of antioxidant therapy to attenuate ischemic reperfusion injury is still unclear (Dhalla *et al.*, 2000).

The cardiac protective role of different antioxidants during reperfusion injury therefore remains inconclusive.

3.5.5. Anti-complement therapy

Tissue damage following reperfusion injury is significantly reduced by complement inhibition in the rat model (C3 convertase inhibitor) (Weisman *et al.*, 1990) as well as humans (C5 inhibition, pexelizumab) undergoing coronary artery bypass graft surgery (Fitch *et al.*, 1999; Shernan *et al.*, 2004). Other trials (Granger *et al.*, 2003; Mahaffey *et al.*, 2003) failed to demonstrate a limitation of infarct-size (total CK-MB) or composite clinical endpoint.

Anti-complement agents are therefore currently undergoing further human clinical trials, and may represent a future therapeutic strategy against reperfusion injury (Eltzschig and Collard, 2004).

3.5.6. Other pharmacologic agents against reperfusion injury

Adenosine has a potential protective effect via better preservation of endothelial function and thus microvascular flow, pharmacological preconditioning and the

inhibition of polymorphonuclear cell activation, ROS formation and neutrophil function (Ely and Berne, 1992). Pre-ischemic administered Adenosine has demonstrated marked cardiac protection in animal studies. Administration *after* ischemia, and for longer ischemic periods, (Babbitt *et al.*, 1990) has however generated variable results (Goto *et al.*, 1991).

The results obtained in conducted clinical trials were mixed and unconvincing in terms of treatment modality against reperfusion injury. The AMISTAD-I and II (Acute Myocardial Infarction Study) trails demonstrated that, although high dose adenosine has infarct size reducing effects, it does not significantly improve clinical outcomes (Ross *et al.*, 2005). This lack in beneficial effects may however be explained by the fact that the studies were underpowered as evidenced by a post hoc analysis which demonstrated a significant clinical improvement in a subset of patients who received adenosine and reperfusion therapy within 3.2 hours of symptom onset (Kloner *et al.*, 2006).

In a randomized placebo controlled trial, intracoronary adenosine administration (following thrombus aspiration and after stenting of the infarct-related artery) did not result in improved myocardial perfusion (Fokkema *et al.*, 2009). Other clinical trials (ADMIRE, n = 608, and the ATTACC, n = 311) also failed to demonstrate beneficial effects in terms of infarct size reduction and clinical outcomes (Kopecky *et al.*, 2003; Quintana *et al.*, 2003). A pilot study (n = 54) done by Marzille *et al.* (2000), however demonstrated less no-reflow, significant improvement in ventricular function (echocardiography) and clinical improvement after intracoronary adenosine administration before reperfusion in patients with acute myocardial infarction (Marzilli *et al.*, 2000).

Taken a collective view at the clinical trials with adenosine, as an adjunct to reperfusion therapy suggests that adenosine failed to show a consistent significant beneficial effect. It may however be of value to further investigate the protective effects of pre-ischemic adenosine administration (Vinten-Johansen *et al.*, 2003; Dirksen *et al.*, 2007).

Glucose-insulin-potassium therapy (GIK) is supposed to be protective during reperfusion injury secondary to:

1. Membrane stabilization (Sodi-Pallares *et al.*, 1962).
2. Glucose being the preferential energy source during ischemia and reperfusion (Fath-Ordoubadi and Beatt, 1997).
3. The reduction in circulating free fatty acid levels and myocardial free fatty acids uptake (Oliver and Opie, 1994).

Studies reported different effects of **glucose-insulin-potassium (GIK) therapy** in terms of major adverse cardiac events and mortality: it ranges between beneficial, (Fath-Ordoubadi and Beatt, 1997; Diaz *et al.*, 1998) no change, (van der Horst *et al.*, 2003; Pache *et al.*, 2004; Mehta *et al.*, 2005; Timmer *et al.*, 2006) and detrimental effects (Ceremużyński *et al.*, 1999).

Despite reports suggesting a beneficial effect on clinical outcome and peri-operative infarct size, GIK is currently not routinely used in clinical practice because of the lack of unequivocally positive results (Doenst, Bothe and Beyersdorf, 2003; Dirksen *et al.*, 2007; Kloner and Nesto, 2008).

The IMMEDIATE clinical trial investigated the effects of GIK therapy, administered out of hospital, to patients experiencing acute myocardial ischemia with suspected acute coronary syndrome. Even though this the study failed to find a difference in the primary endpoint of progression to acute MI, the patients with ST elevation myocardial infarction experienced less cardiac arrest and in-hospital mortality compared to those that received the placebo (Selker *et al.*, 2012). During the one year follow-up, the patients with ST elevation myocardial infarction, demonstrated a significant reduction in the composites of cardiac arrest, 1-year mortality, and heart failure hospitalisation within 1 year (Selker *et al.*, 2014).

Other cardiac protective agents that have been evaluated in the setting of reperfusion injury include **trimetazidine** (Fragasso *et al.*, 2011) (which acts by inhibiting a mitochondrial enzyme and shifts the preference for energy substrate from fatty acid towards glucose metabolism), **angiotensin converting enzyme (ACE)-inhibitors** and **nicorandil** (a NO donor) (Kitakaze *et al.*, 2007). Thus far none of these drugs has been proven to be effective in reducing the infarct size (Dirksen *et al.*, 2007).

The exact role of **calpain inhibitors** in acute myocardial ischemia and reperfusion, although shown to be cardioprotective during myocardial infarction, remains

controversial (Perrin *et al.*, 2003; Neuhof *et al.*, 2004; Neri *et al.*, 2017).

In view of the calcium overload during reperfusion, it was logical to consider **calcium antagonists** as a therapeutic agent against reperfusion injury. Despite promising animal data (Klein *et al.*, 1989; Herzog *et al.*, 1997), the results of clinical trials have been unclear, irrespective of the timing (in relation to ischemia) of calcium antagonist's administration (Sheiban *et al.*, 1997; Théroux *et al.*, 1998; Pizzetti *et al.*, 2001). There is currently no indication for the use of calcium channel blockers as an adjunct to reperfusion therapy, as large randomized trials are lacking (Dirksen *et al.*, 2007). Clinical trials with **$MgSO_4$** (Magnesium sulphate, an endogenous calcium- antagonist) in the setting of acute myocardial ischemia, also showed negative results in over 60,000 patients (Collins *et al.*, 1995).

Another possibility of reducing intracellular calcium overload is to **inhibit sodium-hydrogen exchange**. Studies done with Ranolazine, a sodium hydrogen (Na^+/H^+) exchange inhibitor, unfortunately also failed to improve prognosis (Weisz *et al.*, 2016). Animal studies, as well as some human studies (Boyce *et al.*, 2003; Mentzer, 2003) have however demonstrated a marked decrease in infarct size (enzyme release) when these inhibitors were administered *prior to ischemia*. In general the efficacy of these drugs failed to demonstrate any benefit when given after the onset of ischemia (Klein *et al.*, 2000; Dirksen *et al.*, 2007).

Agents that have been suggested to limit reperfusion injury by **inhibition of the mitochondrial permeability pore (mPTP)** include cyclosporine, (Atar *et al.*, 2014; Cung *et al.*, 2015) exenatide and bendavia. The clinical results are, once again, not encouraging (Ferrari *et al.*, 2017).

In summary: Many therapeutic strategies have been shown to be effective in controlled animal experimental models. Results of clinical studies in terms of cardio protective strategies in humans, have however been ranging from mixed to generally disappointing. The timely reperfusion of the ischemic area at risk therefore currently remains the foundation of clinical practice (Collard and Gelman, 2001).

What is the reason for the failed attempts in translating promising therapeutic strategies into the clinical setting? Contributing factors include (Hausenloy *et al.*,

2010):

- Animal species: The development of a MI differ between a small animals specie, larger animals and humans (Heusch, Boengler and Schulz, 2008; Skyschally *et al.*, 2009).
- Age (Boengler, Schulz and Heusch, 2009): Preclinical experiments are being done in young animals, humans with IHD are usually older and thus potentially resistant to preconditioning (Schulman, Latchman and Yellon, 2001).
- Sex: The majority of animal studies are done in male animals only. Both female and male humans however develop IHD. This may influence the effectiveness of cardioprotective strategies (Ferdinandy, Schulz and Baxter, 2007).
- Co-morbidities: Patients presenting with IHD very often have co-morbid diseases and use chronic medication. This is in contrast to the healthy animals used in studies. IHD self, with repeated episodes of angina, may lead to tolerance against preconditioning. Diabetes, (Sivaraman *et al.*, 2010) and sulphonylureas may prevent cardioprotection as a result of blocking of preconditioning, while nicorandil, ACE-inhibitors, nitrates and statins may have protective effects as a result of inducing preconditioning. Furthermore, patients undergoing anaesthesia may receive drugs (inhalation agents like Sevoflurane and Isoflurane) (Symons and Myles, 2006) which will precondition the heart.
- The type of model used to replicate ischemia: In animal studies regional ischemia is being induced by occlusion of a healthy coronary artery in the absence of collateral perfusion, which is in contrast to what happens in humans. Furthermore, the ischemic and reperfusion time in animal experiments are predetermined, both of which are greatly variable in humans. A successful cardioprotective measure in animal studies might thus be less effective when a longer reperfusion (or ischemic) period is allowed.
- Timing of application of therapeutic intervention: In a multitude of preclinical studies the intervention is being applied pre-ischemic, this is however impractical in the majority of clinical studies.
- The endpoint of cardioprotection: This differs significantly between animals

(infarct size, recovery of contractile function) and humans (biomarkers, imaging and clinical outcome) (Hausenloy *et al.*, 2010).

Other contributing factors include the clinical testing of inconclusive therapies, insufficient dose response studies, technical factors (i.e. techniques for determination of infarct size, handling of the heart, differences between buffered perfusate and blood perfusion with white cells plus inflammatory mediators) and poor design of cardioprotection studies (Becker, 2004; Hausenloy and Yellon, 2013).

CHAPTER 4
PHYSIOLOGICAL ROLE OF AUTOPHAGY DURING ISCHEMIA AND REPERFUSION

4.1. INTRODUCTION

The autophagic process, as discussed previously, is an essential regulating process in the heart under normal conditions. The role of autophagy (pro-death or pro-survival/detrimental or beneficial) during disease processes like ischemia and reperfusion is however still controversial (Shaw and Kirshenbaum, 2008).

The protective and detrimental effects of autophagy on a heart subjected to ischemia and reperfusion has been reviewed by several leading researchers worldwide (Gustafsson and Gottlieb, 2008a; Matsui *et al.*, 2008; Shaw and Kirshenbaum, 2008; Sciarretta *et al.*, 2018).

The significant health and costs implications of ischemic heart disease, as leading cause of mortality and morbidity, and subsequent reperfusion injury were discussed in Chapters two and three. In an attempt to develop therapeutic agents, procedures and treatment strategies against ischemic reperfusion injury, a proper insight into the disease processes and underlying pathology is necessary. This will include the role and different types of myocyte death.

Myocyte death is important in light of the fact that these cells are unique - they are adapted to facilitate lifelong contraction, but also have limited regenerative potential. A dead myocyte can never be replaced – with obvious detrimental biological consequences (Shaw and Kirshenbaum, 2008).

4.2. PATHWAYS INVOLVED IN CELL DEATH

Myocardial cell death might be secondary to four different pathways: necrosis, apoptosis, necroptosis and autophagy; all of which have been found in myocardial cells undergoing ischemia and reperfusion (Chiong *et al.*, 2011).

Necrosis (Greek for: death, causing to die, also known as type 3 cell death) refers to the death of most or all of the cells in an organ or tissue due to disease, injury, or

failure of the blood supply.

Apoptosis (Greek for: falling off, figurative for the falling of leaves, otherwise known as programmed type 1 cell death) is a form of cell death in which a programmed sequence of events leads to the elimination of cells without releasing harmful substances into the surrounding area.

Necroptosis shares some features with both apoptosis and necrosis and is activated by ligands of death receptors. It is a form of programmed cells death (as apoptosis) during which the cell swell, cell fragments are being released during membrane disintegration and an inflammatory response mounted (as during necrosis) (Vandenabeele *et al.*, 2010).

Autophagy can be directly involved in cell death or indirectly by either opposing or enhancing apoptosis (Hamacher-Brady, Brady and Gottlieb, 2006b).

4.2.1. Necrosis

The main causative factor for cell necrosis is physical or chemical cell trauma. Traditionally it was considered to be a passive (non-ATP-dependent) and disordered process, but emerging evidence suggests that there are specific underlying molecular mechanisms and potential genetic elements involved. Proposed mechanisms include death receptors, reactive oxygen species, calcium overload and mitochondrial permeability transition pore (MPTP) opening (Vanlangenakker *et al.*, 2008). The morphological changes characterizing necrosis include cellular swelling secondary to the loss of normal cell regulation processes. This culminates into plasma membrane damage and cell lysis. Disruption of cell integrity followed by the release of degradative cytosolic enzymes consequently trigger an inflammatory response with damage to the neighbouring cells (Tavernarakis, 2007; Shaw and Kirshenbaum, 2008; Chiong *et al.*, 2011).

Myocardial ischemia can induce myocardial necrosis with the necrotic cells centered in the central infarct zone, the extent of the necrosis being determined by the ischemic duration (Matsumura *et al.*, 1998; Hamacher-Brady, Brady and Gottlieb, 2006b).

4.2.2. Apoptosis

Apoptosis is a highly regulated, energy (ATP) dependent process. It plays a role in pathophysiological conditions as well as normal tissue homeostasis, during which this process will get rid of dysfunctional cells. The classic morphological changes include cell shrinkage, membrane blebbing, nuclear and cytoplasmic condensation, and cellular disintegration. All of these morphological changes occur *without* cellular membrane disruption, and therefore the resulting inflammatory response as seen with necrosis (Kerr, Wyllie and Currie, 1972; Shaw and Kirshenbaum, 2008). Instead of global cell membrane disruption the cell is broken into small membrane-enclosed pieces (apoptotic bodies), preventing the release of cellular content.

Caspase activation and *DNA laddering* are two biochemical hallmarks of apoptosis. Caspases, a family of cysteine proteases, are responsible for apoptotic signal transduction. DNA laddering is a consequence of cleaved DNA at the exposed DNA strands secondary to activated nucleases. This results in the creation of DNA fragments (Shi *et al.*, 1990).

In terms of ischemic/reperfusion injury, apoptotic cell death occurs more frequently during reperfusion than during the preceding ischemic period. The dead cells are also more commonly found at the borderline area than the central infarct zone. Apoptosis has been implicated in ischemic reperfusion injury, non-ischemic dilated cardiomyopathy (Schoppet *et al.*, 2005) and congestive heart failure (Olivetti *et al.*, 1997; Hamacher-Brady, Brady and Gottlieb, 2006b).

Apoptosis is mediated by two, evolutionarily conserved and converging pathways, both of which have been described in cardiac myocytes (Chiong *et al.*, 2011). They are known as the extrinsic (receptor-mediated) and the intrinsic (mitochondrial) pathways. The caspase family allow for communication between the two pathways (Hamacher-Brady, Brady and Gottlieb, 2006b).

The **extrinsic pathway** is activated when cell surface death ligands interact with their respective receptor (known as death receptors). In response to the association of the ligands (tumor necrosis factor-alpha, Fas and TNF-Related Apoptosis-Inducing Ligand (TRAIL)) to the receptors, a series of proteins (Death Inducing Signaling Complex (DISC)) are formed. These proteins will undergo biological activation as a result of the

initiator caspase-8 (Shaw and Kirshenbaum, 2008; Chiong *et al.*, 2011).

The **intrinsic pathway** can be initiated by apoptotic triggers including DNA damage, ultra-violet radiation, loss of growth factors, oxidative stress, drugs, toxins and hypoxia. The cells sense these harmful signals and induce a variety of changes including the activation of death genes and the suppression of survival genes. This apoptotic process is facilitated by energy supplied from the mitochondria. The resulting release of pro-apoptotic protein cytochrome c results in caspase-9 activation, which in turn activates effector caspases.

The Bcl-2 protein family is important in the intrinsic pathway and possesses both anti-apoptotic (Bcl-2 itself, Bcl-X_L and Bcl-w) as well as pro-apoptotic family members (Bax, Bak, Bid, Bad, Puma, Noxa and Bnip3). The “apoptotic fate” will be determined by the cellular ratio between the pro- and anti-apoptotic Bcl-2 proteins (Zou *et al.*, 1999; Gustafsson and Gottlieb, 2007; Shaw and Kirshenbaum, 2008).

4.2.3. Necroptosis

This recently discovered mechanism of cell death, programmed necrosis (necroptosis), combines features of both necrosis and apoptosis, as mentioned earlier (Zhe-Wei, Li-Sha and Yue-Chun, 2018).

While necrosis is considered to be an accidental or passive type of cell death, apoptosis is a highly modulated, systematically process whereby damaged cells are eliminated. This new form of *programmed* necrosis, involves active cell death triggers (RIP1/3, receptor-interacting protein 1/3) by specific signaling pathways rather than non-specific injury (Vandenabeele et al., 2010).

Recently, there has been increasing evidence showing that necroptosis plays an important role in cell death during reperfusion injury. Myocardial reperfusion injury can trigger necroptosis via two pathways, RIP3-CaMKII (Calmodulin-dependent protein kinase II) and RIP1-RIP3-MLKL (mixed lineage kinase domain-like). This then leads to mPTP opening, an increase in oxidative stress and an inflammatory response initiating myocyte loss and thus contributing to reperfusion injury. Necrostatin-1, an RIP1 inhibitor, may be a future therapeutic agent against myocardial reperfusion injury. (Oerlemans *et al.*, 2012; T. Zhang *et al.*, 2016; Zhe-Wei, Li-Sha and Yue-Chun,

2018; Zhu *et al.*, 2018).

4.2.4. Autophagic cell death

Autophagy (Greek for: self-digestion), acts as a pro-survival program under normal conditions by maintaining cellular homeostasis, acting as an energy source and counteracting apoptotic cell death under most conditions (Boya *et al.*, 2005; Hamacher-Brady, Brady and Gottlieb, 2006b; Ravikumar *et al.*, 2006).

Autophagy, however, has also been implicated as a pro-death pathway in the heart. Studies have shown that autophagy can act as an alternative form of programmed cell death, termed “autophagic cell death” (ACD) or Autosis (Shen, Kepp and Kroemer, 2012) is also referred to as programmed cell death (PCD) type II. The Nomenclature Committee on Cell Death (NCCD) describe autophagic cell death as cell death that is “suppressed by inhibition of the autophagy pathway” (Galluzzi *et al.*, 2012). Inhibition of autophagy, by 3 Methyl-adenine (3MA), prevents death of H9c2 cells in vitro (Aki *et al.*, 2003) while extensive autophagy is often seen in dying cells. This cell death pathway, which will be discussed in the next section, is thought to be distinct from apoptosis and might be the consequence of constitutive and excessive autophagy (Bursch, 2001; Hamacher-Brady, Brady and Gottlieb, 2006b; Gustafsson and Gottlieb, 2008a; Ma *et al.*, 2015).

The functional contribution, and the true existence of autophagic cell death has however been a subject of debate. The question is whether increased autophagy in dying cells is the “cause of cell death or a failed attempt to prevent it” (Matsui *et al.*, 2007). “Autophagic cell death” insinuates that cell death is a direct consequence of autophagy, and an extensive literature review (done before 2008) by Shen *et al.* (2011) could not find any supporting evidence for true Autophagic cell death in mammalian cells (Shen *et al.*, 2011; Shen, Kepp and Kroemer, 2012). Kroemer and Leviine (2008) therefore view “autophagic cell death” as a misnomer since it, thus far, describes a “reality in which cells die with autophagy but not by autophagy” (Kroemer and Levine, 2008; Shen, Kepp and Kroemer, 2012).

The mechanisms of indirect autophagic cell death might involve interaction with apoptotic (Hamacher-Brady, Brady and Gottlieb, 2006b) and necrotic pathways (Shen *et al.*, 2011).

Two possibilities for autophagy being responsible for cell death therefore exist (Figure 4.1). (A) Autophagy causing cell death directly, and thus being responsible for the destruction of cellular content. (B) Autophagy being indirectly responsible for cell death by manipulation and/or activation of apoptotic or necrotic death pathways.

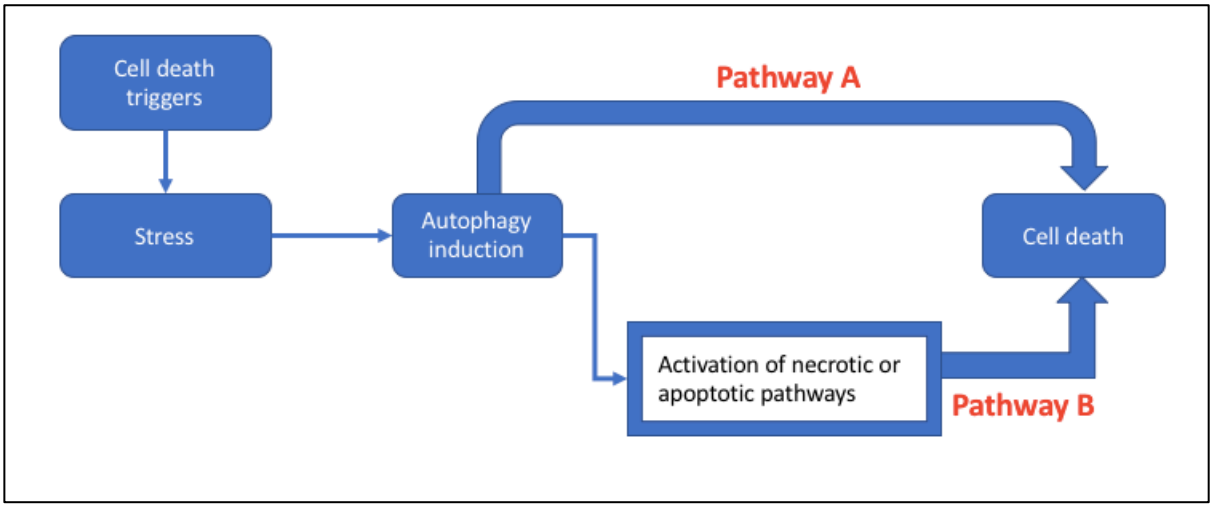


Figure 4.1: Different pathways by which autophagy may cause cell death

Pathway A represent autophagy directly causing cell death, and pathway B autophagy indirectly responsible for cell death

(Adapted from Shen, Kepp and Kroemer, 2012)

4.2.5. Autosis

In addition to autophagy related cell death, described above, Liu *et al*. (2013) described autosis, an autophagy-dependent non-apoptotic form of cell death (Liu *et al.*, 2013).

The same author ascribes the following criteria to autosis (Liu and Levine, 2014):

- The absence of (morphological, biochemical and genetic) evidence supporting other cell death pathways.
- Specific morphological characteristics, and
- A unique dependence on Na^+K^+ ATPase.

The morphologic features include enhanced cell substrate adhesion, late disappearance of ER secondary to fragmentation and dilatation, accumulation of autophagosomes and autolysosomes, nuclear membrane convolution and focal swelling of the perinuclear space (Liu *et al.*, 2013). The nuclear membrane changes,

namely nuclear shrinkage with a focal concave part bordering a balloon-like entity, are viewed as most important.

Pharmacological inhibition or genetic inactivation of Na^+K^+ ATPase blocks autosis in vitro and in vivo, whereas inhibitors of apoptosis and necrosis are without effect. It is induced by autophagy inducing peptides, autophagic activators (TAT-Beclin 1) starvation and hypoxia-ischemia (Liu *et al.*, 2013).

The questions - if autosis only exist as a subtype of autophagy related cell death, its biological and clinical implications as well as the existence of specific biomarkers – remain largely unanswered (Sciarretta *et al.*, 2018).

4.2.6. Interaction (crosstalk) between the autophagy and apoptotic pathways

Autophagy and apoptosis are both recognized pathways for cell death. Autophagy and apoptosis however also share molecular regulators, which include the anti-apoptotic and pro-apoptotic members of the Bcl-2 family proteins as well as Atg5 (Hamacher-Brady, Brady and Gottlieb, 2006b).

- Pro- and anti-apoptotic Bcl-2 family members as regulators of autophagy

Traditionally Beclin-1 is known for the central role it plays in autophagy and the Bcl-2 protein family is involved with apoptosis. Beclin1 is however also a Bcl-2 interacting protein, the Bcl-2 binding domain of Beclin1 serves as a point of communication between the autophagic and apoptotic pathways. This binding domain on Beclin however only exist for anti-apoptotic Bcl-2 family members (Liang *et al.*, 1999; Hamacher-Brady, Brady and Gottlieb, 2006b). **Beclin-1** therefore also has anti-apoptotic properties, which may be ascribed to the interaction it has with the anti-apoptotic proteins **Bcl-2** and **Bcl-X_L** (Shaw and Kirshenbaum, 2008).

The autophagic role of these anti-apoptotic proteins are controversial. Shimizu and Brady *et al*. suggested a pro-autophagic role, (Shimizu *et al.*, 2004; Brady *et al.*, 2007) while others provided contrary evidence (Pattingre *et al.*, 2005; Criollo *et al.*, 2007). Brady *et al*. made use of autophagic “flux” to measure the amount of autophagy, and he attributed the discrepancies between the studies secondary to the different autophagy measurement techniques (Brady *et al.*, 2007).

From a pure cardiac point of view, apoptosis is regarded as a contributor to heart failure in human hearts (Narula *et al.*, 1996; Olivetti *et al.*, 1997). Studies have shown that stimulating apoptosis will induce heart failure while inhibition of apoptosis reduces heart failure and improves survival (Hayakawa *et al.*, 2003).

The pro-apoptotic Bcl-2 family member **Bnip3** (Bcl-2/Adenovirus E1B 19 kDa Protein-Interacting Protein 3) plays an important role in above mentioned process, since it is upregulated in myocytes secondary to hypoxia (Kubasiak *et al.*, 2002; Regula, Ens and Kirshenbaum, 2002). A study done on Bnip3 deficient mice undergoing ischemic reperfusion injury, implied a protective, anti-apoptotic effect, (Diwan *et al.*, 2007) whereas an elevated Bnip3 expression in controls exposed to ischemia had larger infarcts (and increased apoptosis).

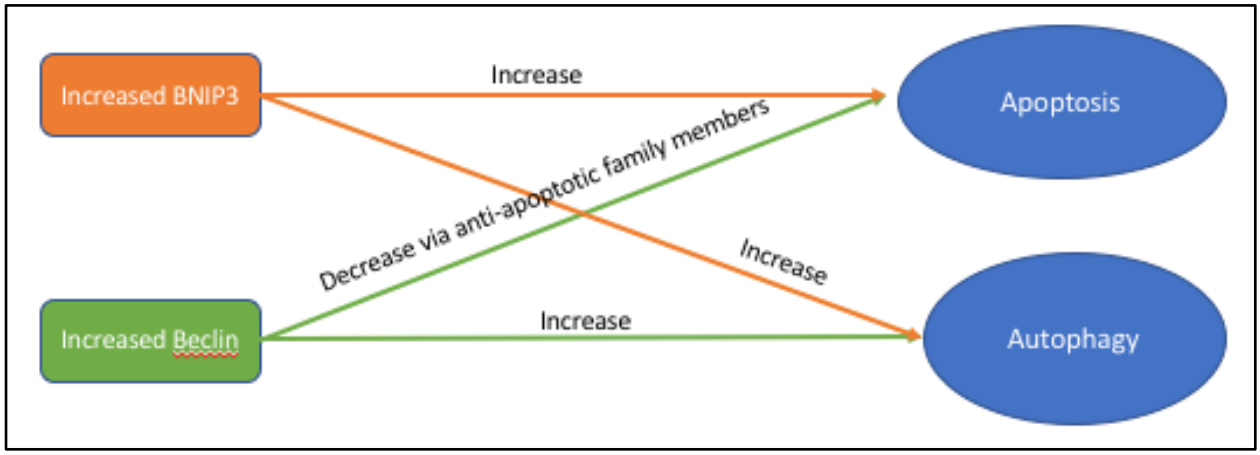


Figure 4.2: Illustration of the crosstalk between pro-apoptotic Bcl-2 family member, Bnip3, and Beclin in terms of apoptosis and autophagy

It has been implicated that Bnip3 might furthermore be responsible for a caspase-independent, necrosis-like cell death, (Vande Velde *et al.*, 2000) as well as autophagy induction (Hamacher-Brady *et al.*, 2007). The role of cell death secondary to Bnip3 may be context specific, and more research is needed clarify the role of Bnip3 in apoptosis and autophagy.

There is also evidence for apoptotic and autophagic crosstalk between the pro-apoptotic proteins Bax and Bid (Kågedal *et al.*, 2005; Lamparska-Przybysz, Gajkowska and Motyl, 2005).

It is clear that a dynamic relationship exists between apoptosis and autophagy, and that it is, at least partially, connected by the Bcl-2 family members.

- Atg 5 (autophagy related gene) as a regulator of apoptosis

There is ample evidence to support the role of Atg5 in *both* autophagy and apoptosis. Today Atg5 (as part of the ATG12-ATG5:ATG16L complex) is known to be a key protein responsible for elongation of the phagophore (the beginning of the autophagosome), as discussed in Chapter 1. Atg5 was however initially described as an apoptosis specific protein, and was found to be upregulated during apoptosis (Masaki *et al.*, 2000; Hamacher-Brady, Brady and Gottlieb, 2006b).

Calpain 1 and 2-mediated cleavage of Atg5 enables the translocation of cleaved Atg5 from the cytosol to the mitochondria, (Yousefi *et al.*, 2006) where it induces cytochrome C release and therefore apoptosis via the intrinsic pathway (similarly to the pro-apoptotic Bcl-2 family member proteins). A potential secondary apoptotic mechanism exists whereby Atg5 acts as an antagonist of Bcl-X_L (an anti-apoptotic protein) (Shaw and Kirshenbaum, 2008). A recent study reported that Atg5 can also activate extrinsic apoptotic pathways via the interaction with FADD, and possibly caspase 8 (Pyo *et al.*, 2005). (Refer to Figure 4.3)

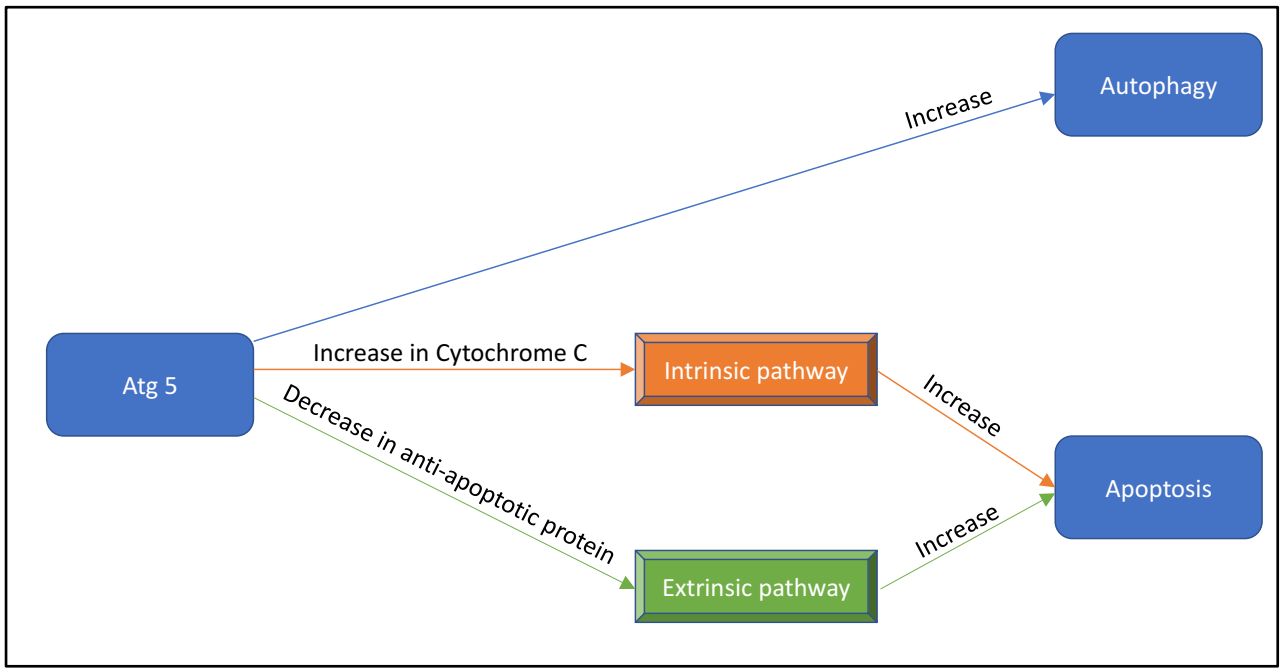


Figure 4.3: The roles of Autophagy related gene 5 (Atg5) in autophagy and apoptosis

Caspase 8 (known for its pro-apoptotic function) may, on the other hand, be responsible for the suppression of autophagic cell death (Hamacher-Brady, Brady and Gottlieb, 2006b).

Timing also seems to play an important role in this crosstalk relationship between autophagy and apoptosis. Yan and colleagues demonstrated early activation of apoptosis in a chronic, repetitive ischemia model. Apoptosis furthermore peaked after three ischemic episodes and there was a significant reduction after six episodes. Autophagy was only observed after three ischemic episodes, and it peaked around the sixth episode (Yan *et al.*, 2005, 2006). Matsui *et al*. (2007) however found that autophagy appeared before apoptosis, which was observed mostly in the reperfusion phase (Matsui *et al.*, 2007).

The relationship between autophagy and apoptosis is clearly complicated. Autophagy can inhibit (Bauvy *et al.*, 2001; Yan *et al.*, 2005; Nishida, Yamaguchi and Otsu, 2008; Long *et al.*, 2013) and induce (Xu *et al*., 2013) apoptosis. In a study done on cardiomyocytes during ischemia and reperfusion, Rapamycin (an autophagic inducer) was found to promote cell survival, increased autophagy and inhibited apoptosis. In the same study Wortmannin (an autophagic inhibitor) was responsible for an increase in apoptotic cell death. This might suggest that autophagic induction has cardioprotective effects as a consequence of apoptotic inhibition. These effects were however ischemic-time sensitive – with a prolonged ischemic (24 hours) time the protective effects of Rapamycin was lost because *both* apoptotic and autophagic cell death (autosis) were significantly increased. Wortmannin, on the other hand, increased survival by inhibiting the excessive autophagic upregulation during prolonged ischemia (Q. Xu *et al.*, 2015). Yan *et al*. (2006) however found autophagy to play a protective role by means of apoptotic inhibition *even* in chronic ischemia. The model used in this study was pigs and chronic ischemia was defined as 6 episodes of 90min of coronary occlusion, an accumulative ischemic time of 9 hours (Yan *et al.*, 2006). Ravikumar *et al*. (2006) also referred to the protective effect of autophagic induction (also done with Rapamycin) as a result of enhanced mitochondrial clearance caused by a reduction in cytochrome c and caspase release and therefore less apoptosis (Ravikumar *et al.*, 2006).

(The complicated interaction between apoptosis and autophagy will be further discussed and referred to in the discussion, Chapter 7, Section 7.7.)

4.3. PUTATIVE ROLES OF AUTOPHAGY IN DIFFERENT CONDITIONS

Under **normal conditions** autophagy is an important and continuous housekeeping process in the cells. It is responsible for nutrient homeostasis and serves as an energy source, during starvation, via the degradation and removal of old and malfunctioning cytosolic components (Hamacher-Brady, Brady and Gottlieb, 2006b; Gustafsson and Gottlieb, 2008a).

Autophagy is therefore rapidly activated during **starvation or stress** (Levine and Klionsky, 2004) during which it acts as a survival mechanism (J. Zhu *et al.*, 2007). The autophagosome removes damaged organelles and uses the products of degradation to maintain mitochondrial ATP production and protein synthesis (Matsui *et al.*, 2007; Gustafsson and Gottlieb, 2008a).

In contrast to above-mentioned pro-survival aspects, autophagy can also initiate (type II) cell death in different circumstances, as mentioned earlier (Thapalia, Zhou and Lin, 2014). A **dysregulated autophagic response** may contribute to a multitude of diseases and disorders such as neurodegenerative diseases, (Komatsu *et al.*, 2006) cancer, (S. Yang *et al.*, 2011) liver diseases, (Hidvegi *et al.*, 2010) metabolic syndromes, (Codogno and Meijer, 2010) ageing, (Rubinsztein, Mariño and Kroemer, 2011) and inflammation (Virgin and Levine, 2009). Other examples of “defective autophagy” include Huntington’s disease, Parkinson’s disease, Alzheimer’s disease, Lafora disease, hepatocellular carcinoma, hepatitis, fibrosis, diabetes, obesity, pancreatitis, infectious diseases, Pompe’s Disease and Crohn’s Disease (Thapalia, Zhou and Lin, 2014).

Increasingly evidence suggests that autophagy plays a prominent role in **cardiovascular diseases** including cardiac hypertrophy, (Dämmrich and Pfeifer, 1983) cardiomyopathy (Tanaka *et al.*, 2000) and heart failure (Pfeifer *et al.*, 1987; Nishino *et al.*, 2000; Shimomura *et al.*, 2001; H. Zhu *et al.*, 2007). The question remains whether the upregulation of autophagy during these conditions is a cardio-protective (Hamacher-Brady, Brady and Gottlieb, 2006b; Nakai *et al.*, 2007) or maladaptive (Valentim *et al.*, 2006; H. Zhu *et al.*, 2007) response to cardiac injury

(Iwai-Kanai *et al.*, 2008).

Cardiomyocytes have little regenerative capacity and is therefore very vulnerable. The low-level, self-digestive process of cardiac autophagy under normal conditions contributes to cellular homeostasis through clearance of excessive or damaged proteins. The *protective role* of cardiac autophagy is therefore being demonstrated by the role it plays in the maintenance of cardiac structure protein and organelle quality control. This is supported by the fact that the loss of essential autophagic mediator molecules (LAMP2 and Atg5) result in cardiac dysfunction (Ma *et al.*, 2015).

Decreased levels of LAMP2 (lysosome associated membrane protein), a membrane protein important for autophagosome-lyosome fusion, are the cause of Danon disease - a condition characterized by severe and progressive myopathy, mental retardation, cardiomyopathy and left ventricular dilation and dysfunction (Saftig *et al.*, 2001; Maron *et al.*, 2009). The defect in terms of autophagosome-lysosome fusion results in autophagosome accumulation, which interferes with contractile function (Saftig *et al.*, 2001). These patients experience severe, often fatal, cardiomyopathy due to this autophagic pathway defect, which suggest that autophagy is a routine and beneficial cellular process in the heart (Shaw and Kirshenbaum, 2008; Ma *et al.*, 2015).

A study done by Nakai *et al.* (2007) also suggests that autophagy plays a protective role in the heart during stressed conditions. Hearts from two groups (Atg5 deficient and control mice) were subjected to pressure overload by aortic constriction. The Atg5 deficient group demonstrated less autophagy, more ventricular dilatation and contractile dysfunction, similar hypertrophic response to pressure overload and an increase in apoptosis in comparison to the control group (Nakai *et al.*, 2007). H. Zhu *et al*. (2007) however found the opposite, the conclusion of their research involving Beclin1 overexpression was that cardiac autophagy is a maladaptive response to hemodynamic stress in the form of pressure overload (H. Zhu *et al.*, 2007). The different outcomes of these studies might be ascribed to the different autophagic proteins used to target autophagy (Atg5 vs. Beclin1), differences in the mice used, the degree of pressure overload applied and the specific points in time used for measurements (Nakai *et al.*, 2007).

Two other (hamster) studies investigating the role of autophagy in the context of

dilated cardiomyopathy both found that apoptosis was not the major mode of cell death in a model of non-ischemic cardiomyopathy. Miyata *et al*. (2006) ascribed this role to autophagy while Ryoke *et al.* (2002) found that necrosis was the dominant contributor to cell death (Ryoke *et al.*, 2002; Miyata *et al.*, 2006).

Unlike the above mentioned studies, apoptotic cell death was reported to play a dominant role in the disease progression in dilated cardiomyopathy in humans (Narula *et al.*, 1996; Olivetti *et al.*, 1997). These differences may be a reflection of the dissimilarities between different models (human and hamster), but it also highlights the need for simultaneous investigations into apoptosis and autophagy as mechanisms of cell death (Shaw and Kirshenbaum, 2008).

A number of studies support the detrimental effect of autophagy (in the form of autophagic cell death) in the failing heart. An increase in autophagosomes are found in cardiomyopathy, (Shimomura *et al.*, 2001; Saijo *et al.*, 2004; Miyata *et al.*, 2006) as a result of myocardial hibernation (Elsässer *et al.*, 2004) and Diphtheria toxin-activated myocardial cell death (Akazawa *et al.*, 2004).

Taking a collective look at above, the role of autophagy in cardiovascular disease is noticeably controversial.

In summary: Autophagy clearly fulfil different roles in different conditions:

- Under normal conditions basal levels of autophagy are important for maintenance of cellular homeostasis and cell protection.
- An increase in autophagy may be beneficial in terms of survival in response to stress and starvation, whereas extreme and longstanding (dysregulated) upregulation of autophagy may be responsible for cell death and therefore detrimental (Matsui *et al.*, 2007; Gustafsson and Gottlieb, 2008a).
- The autophagic role is not similar for different cardiac conditions, the detrimental vs protective effect may be model and situation specific (Shaw and Kirshenbaum, 2008).

4.4. AUTOPHAGIC RESPONSE TO HYPOXIA AND ISCHEMIA-REPERFUSION

4.4.1. Ischemia

Multiple studies demonstrated that autophagy is upregulated in response to ischemia (and continues or even increases during the later stages of reperfusion) (Kloner and Jennings, 2001). This upregulation was reported in neonatal cardiomyocytes from mouse hearts, (Sybers, Ingwall and DeLuca, 1976; Q. Xu *et al.*, 2015) Langendorff perfused rabbit hearts, (Decker and Wildenthal, 1980) a swine model, (Yan *et al.*, 2005) isolated cardiac myocytes (Hamacher-Brady, Brady and Gottlieb, 2006a; Q. Xu *et al.*, 2015) and the mouse heart in vivo (Matsui *et al.*, 2007). Ischemia/hypoxia however does not induce autophagy in all studies. This was illustrated by French *et al*. (2010) who found that autophagy is virtually absent in the center of the infarct zones, and decreased significantly in the peri-infarct zones, when compared to normal in vivo mouse hearts (French, Taatjes and Sobel, 2010).

It appears that the ischemic and reperfusion duration plays a significant role. In rabbit hearts 20 minutes of ischemia did not induce autophagy, the number of autophagosomes however increased after initiation of reperfusion. Prolonging the period of ischemia (to 40 minutes) caused an increase in autophagy, which was further enhanced during reperfusion. After 60 minutes of ischemia autophagic flux appeared impaired in these rabbit hearts (Decker and Wildenthal, 1980). In mouse hearts, however, autophagy was already present within 20 minutes of ischemia and it persisted during the reperfusion phase. The onset of the appearance of autophagy seems faster according some studies, (Matsui *et al.*, 2007) and slower according to other (Yan *et al.*, 2005) than that of apoptosis. Autophagy may serve primarily to maintain energy production during acute ischemia but switch to clearing up damaged organelles during chronic ischemia or reperfusion (Matsui *et al.*, 2007).

It appears that the ischemic duration/degree in combination with the experimental model and the specie will determine the presence versus the absence of autophagic upregulation in response to ischemia (Przyklenk *et al.*, 2012).

The two main pathways responsible for autophagic induction during ischemia include:

- AMPK activation

The cardiac myocyte relies heavily on mitochondrial oxidative phosphorylation for energy. Myocardial ischemia is followed by a rapid decline in ATP and increased AMP/ ATP ratio (Chiong *et al.*, 2011). These changes are responsible for the activation of AMPK (adenosine monophosphate-activated protein kinase) – a sensitive sensor of cellular energy. AMPK on its turn will induce autophagy via mTOR inhibition and ULK1 activation, as discussed in Chapter 1 (Refer to Chapter 1, Figure 4) (Matsui *et al.*, 2007; Kim *et al.*, 2011).

- Hypoxia-inducible factor 1 alpha (HIF-1α)

Hypoxia (ischemia) can activate HIF-1, which has been shown to induce BNIP3 expression. The pro-apoptotic protein BNIP3 can regulate the expression of autophagy-related genes including Beclin1 and Atg5 (H. Zhang *et al.*, 2008).

The over-expression of BNIP3 will result in Bcl-2/Beclin1 or Bcl-X /Beclin1 complex dissociation, and is responsible for the up-regulation in Beclin1 expression, which is capable of triggering autophagy, as discussed in Chapter 1 (Bellot *et al.*, 2009; Zhao *et al.*, 2012; Russell *et al.*, 2013).

4.4.2. Reperfusion

The majority of evidence also points to an upregulation of autophagy during reperfusion, this include studies done in rats,(Huang *et al.*, 2010) rabbits, swine hearts(Yan *et al.*, 2005) and cardiomyocytes (Hamacher-Brady, Brady and Gottlieb, 2006a; Valentim *et al.*, 2006; Matsui *et al.*, 2007; Gatica *et al.*, 2015).

This continuation of autophagic upregulation during reperfusion take place irrespective of the re-establishment of oxygen supply and therefore inactivation of AMPK. The underlying mechanism for autophagic induction during reperfusion is therefore different from that in ischemia (Chiong *et al.*, 2011). Endoplasmic reticulum and oxidative stress in combination with mitochondrial damage, BNIP3 and calcium overload play important roles in maintaining autophagy at a higher level during reperfusion (Gustafsson and Gottlieb, 2009).

As mentioned, during reperfusion (the re-introduction of oxygen), AMPK is no longer activated and mTOR is no longer inactive. Thus, the AMPK pathway is therefore not the major mediator of autophagy induction during reperfusion. Matsui demonstrated in 2007 that myocardial ischemia stimulates autophagy through AMPK- and Beclin 1–dependent mechanisms, and that ischemic reperfusion injury stimulates autophagy through Beclin 1–dependent mechanisms only. Matsui *et al*. (2007) therefore proposed that the AMPK-dependent signaling mechanism mediates autophagy during ischemia, whereas in the reperfusion phase, *Beclin 1* plays an essential role in mediating the (mTOR-independent but Beclin1–dependent) autophagy process (Matsui *et al.*, 2007).

The question is, how does reperfusion injury activates Beclin1? Possibilities include Beclin1's association with Bcl-2 protein (Brady *et al.*, 2007) and the reactive oxygen species (ROS) (Hariharan, Zhai and Sadoshima, 2011) released during the reperfusion process (Ma *et al.*, 2015) (Refer to Figure 4.4).

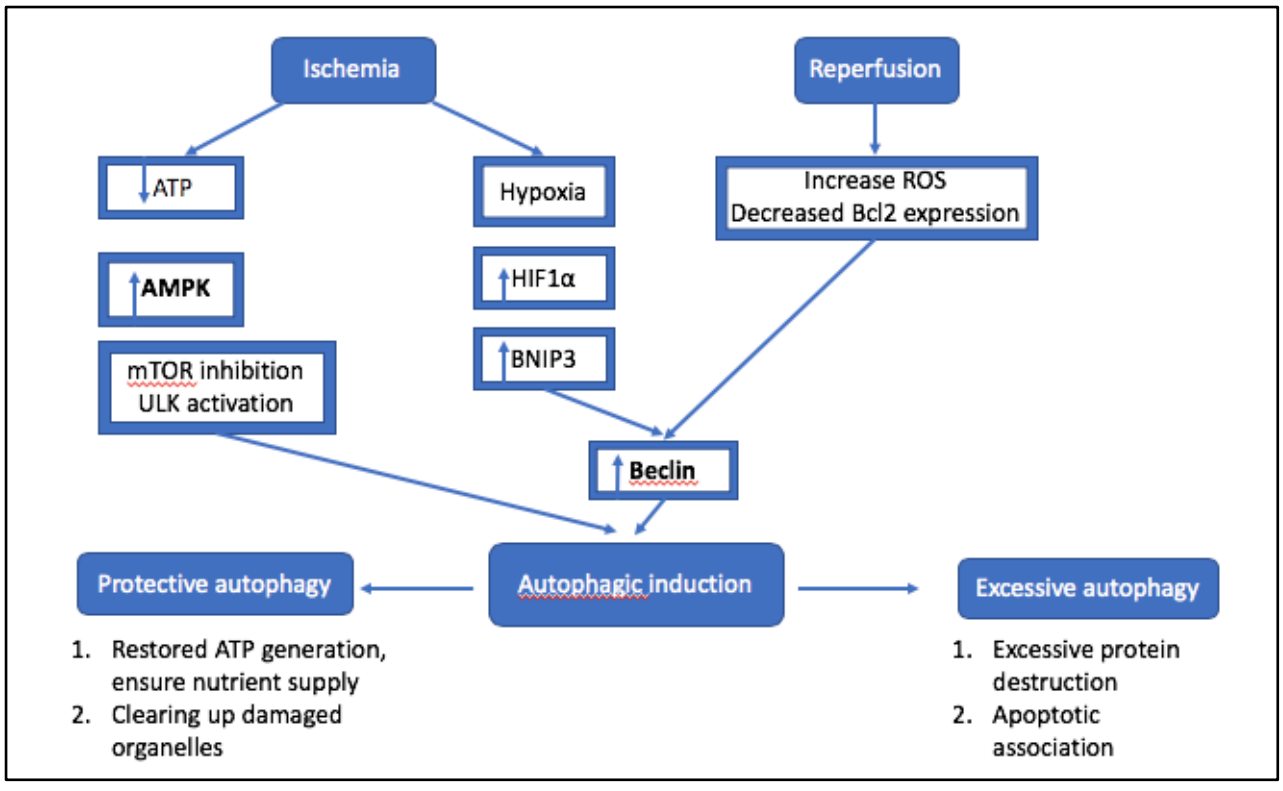


Figure 4.4: Autophagic response, as well as mechanisms involved, during ischemia and reperfusion injury
Source: Adapted from Ma *et al.*, 2015.

4.4.3. The role of autophagic flux

Autophagic flux, as alluded to before, refers to the dynamic process of autophagosome formation, followed by lysosomal fusion and the subsequent degradation of the autophagolysosome (Loos, Du Toit and Hofmeyr, 2014). Increased formation (accumulation) of autophagosomes may therefore be secondary to an increase in production, or a decrease in clearance. It is therefore important to establish the precise cause for an increase in autophagosomes/autophagy – to be able to distinguish between a true increase in autophagy (autophagic flux) and a decrease in clearance (Ma *et al.*, 2015). Since 'autophagy' is a process, dependence on a single intermediate (in the form of autophagosomes) may not be the most precise way to assess efficiency.

There are conflicting reports in terms of the efficiency of autophagy (autophagic flux) during ischemia and reperfusion. Some studies supporting an increase in autophagic flux during ischemia (Zhang *et al.*, 2014; Q. Xu *et al.*, 2015) and reperfusion (Hariharan, Zhai and Sadoshima, 2011; Orogo and Gustafsson, 2015). Other studies support the presence of the inhibition of autophagic flux during prolonged ischemia (Orogo and Gustafsson, 2015) and during reperfusion (Ma, Liu, Foyil, Godar, Weinheimer and Diwan, 2012; Ma, Liu, Foyil, Godar, Weinheimer, Hill, *et al.*, 2012). It appears as if the duration and the degree of ischemia and reperfusion, the experimental model used and the manner in which autophagy (and flux) were measured all play an important role (Sciarretta *et al.*, 2011).

Ma *et al.*, supporting a partial inhibition of autophagy flux during ischemia and severe impairment during reperfusion, proposed the following mechanisms for the impairment in autophagic flux.

- A decline in levels of LAMP2, induced by both ischemia and reperfusion. LAMP2 is critical for autophagosome-lysosome fusion.
- Beclin-1 abundance during reperfusion, with resulting autophagosome accumulation. This may play a role in determining the end result of autophagy i.e. efficient autophagic degradation contributing to cellular homeostasis, or autophagosome accumulation prompting programmed cell death (Ma, Liu, Foyil, Godar, Weinheimer, Hill *et al.*, 2012).

Both above mentioned processes are driven by reactive oxygen species generated during reperfusion (Ma, Liu, Foyil, Godar, Weinheimer and Diwan, 2012). The inhibition of autophagic flux during ischemic reperfusion injury, is in opposition to the conventional view of further upregulation of autophagy during reperfusion (Ma *et al.*, 2015).

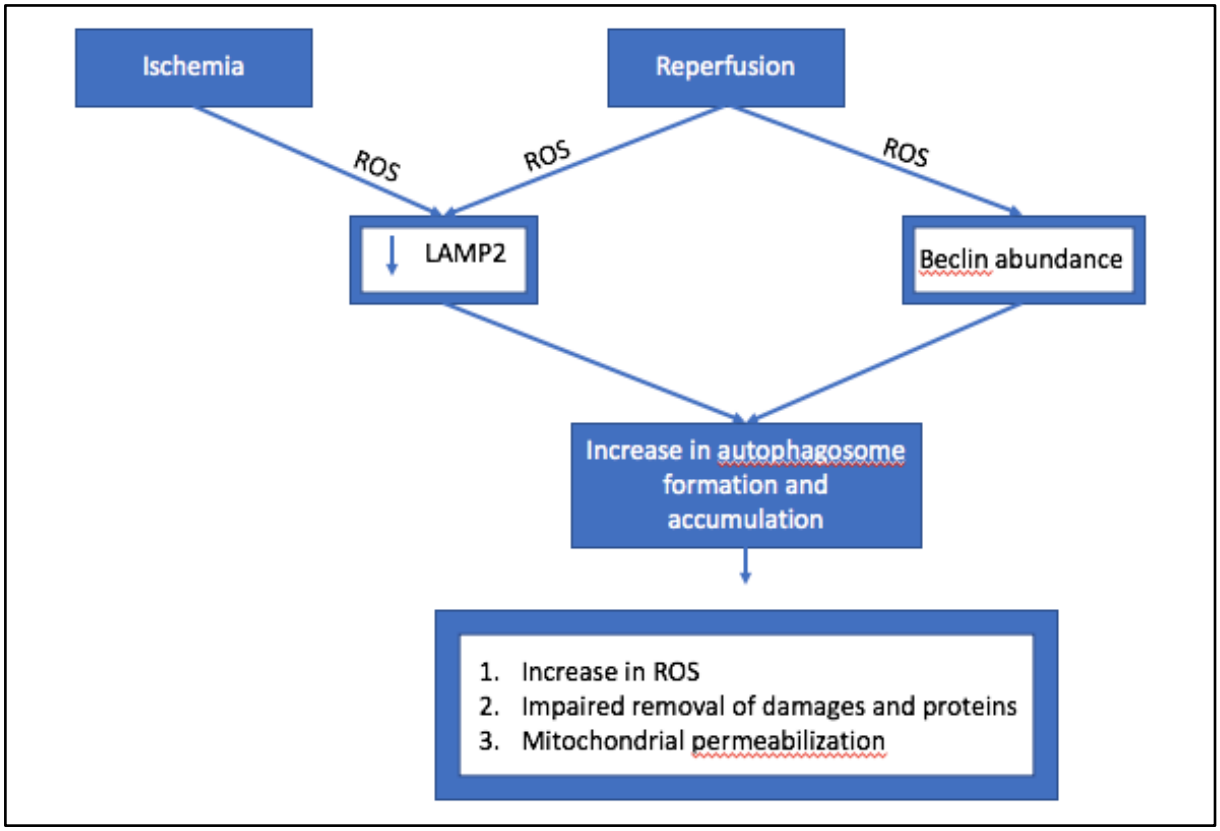


Figure 4.5: Mechanism for, and consequences of, autophagic flux inhibition during ischemia and reperfusion
Source: Adapted from Ma, Liu, Foyil, Godar, Weinheimer and Diwan, 2012.

Autophagosome accumulation results in an increase in reactive oxygen species levels, which may activate mitochondrial permeability and contribute to cell death (Ma, Liu, Foyil, Godar, Weinheimer, Hill *et al.*, 2012).

Angiotensin II (Ang II) receptor signaling also plays a role in autophagic induction during myocardial ischemia and reperfusion. Angiotensin II stimulation of the Angiotensin I receptor is responsible for an increase in autophagy, whereas overexpression of Angiotensin II receptor can inhibit autophagy (Dai *et al.*, 2010). This led to the conclusion that Angiotensin I receptor signaling may be involved in autophagic induction during myocardial ischemia and reperfusion (Zhang *et al.*, 2013).

In summary,

- The majority of evidence point towards an increase in autophagosome formation during the ischemic as well as reperfusion period.
- The degree of autophagic induction might be influenced by the duration and the degree of ischemic reperfusion injury, as well as the (animal) model used (Sadoshima, 2008).
- AMPK, Beclin1, Bcl-2 family members and angiotensin II/AT1 receptor signaling may all be involved in the stimulation of autophagy during ischemia and reperfusion in the heart.
- The underlying mechanisms for autophagy induction during ischemia and reperfusion are different (Matsui *et al.*, 2007).
- This increase in autophagosomes is not a representation of autophagic flux, and the influence of ischemic reperfusion injury on autophagic flux is still controversial.

4.5. CONSEQUENCES SECONDARY TO AUTOPHAGIC CHANGES IN ISCHEMIA AND REPERFUSION

It appears that the effects of autophay differ according to the conditions studied. As discussed before - autophagy plays an adaptive role during baseline conditions (Sciarretta *et al.*, 2011) and has a protective role during starvation (Levine and Yuan, 2005). The role of autophagy in in myocardial ischemia/reperfusion injury (beneficial vs detrimental) is however still a subject of debate (Hamacher-Brady, Brady and Gottlieb, 2006a; Valentim *et al.*, 2006). The limitations in the ability to accurately measure autophagic flux (as alluded to before) also contribute to this controversy (Aki *et al.*, 2003; Gustafsson and Gottlieb, 2008a; Iwai-Kanai *et al.*, 2008).

With the current conflicting available knowledge, autophagy can be viewed as a double-edged sword in the pathological process of I/R injury (Ma *et al.*, 2015).

4.5.1. Support for the beneficial consequences following induction of autophagy

The majority of studies support the hypothesis that an upregulation of autophagy, in response to ischemic reperfusion injury, is indeed cardio-protective (Dosenko *et al.*, 2006; Hamacher-Brady, Brady and Gottlieb, 2006a; Valentim *et al.*, 2006; Yan *et al.*, 2006; Matsui *et al.*, 2007; Iwai-Kanai *et al.*, 2008; Kanamori *et al.*, 2011; Sciarretta *et al.*, 2011; Q. Xu *et al.*, 2015).

Studies suggesting a myocardial protective effect secondary to the induction of autophagy during ischemia and reperfusion injury include:

- Hariharan *et al*. (2011) showed that augmentation of autophagic flux during ischemia and reperfusion has a protective effect on cardiomyocytes in vitro (Hariharan, Zhai and Sadoshima, 2011).
- In pigs, pharmacological induction of autophagy was accompanied by a reduction of apoptosis in myocardial cells and a reduction of myocardial injury (Sala-Mercado *et al.*, 2010).
- Induction of autophagy, in rabbit hearts, assist in the myocardial repair efforts, and therefore prevent significant myocardial injury, during prolonged ischemia (Decker and Wildenthal, 1980).
- In the pig heart, it was demonstrated that significant autophagic upregulation following chronic ischemia, inversely correlated with apoptotic activity in the ischemic area. In this model, the ischemic area showed full recovery with minimal evidence of cell death after relief of hypoxia. When endogenous AMPK was inhibited (and autophagy suppressed), during prolonged ischemia, there was a substantial increase in infarct size (Yan *et al.*, 2005).
- The above observation was confirmed in the mouse model by Takagi *et al*. (2007) They also found that endogenous AMPK inhibition during prolonged ischemia was responsible for myocardial infarction enlargement (Takagi *et al.*, 2007).
- A study done in cardiac myocyte-like HL1 cells demonstrated that the pharmacological enhancement of autophagy (by Rapamycin) reduced apoptosis

after ischemia and reperfusion and had a protective effect on ischemic reperfusion injury (Hamacher-Brady, Brady and Gottlieb, 2006a).

Potential mechanisms for the cardio protective effect following autophagic induction during ischemia (Matsui *et al.*, 2008).

1. Autophagy as a source of energy during hypoxia

Sufficient ATP supply is an essential requirement for cardiomyocytes to function normally. During ischemia ATP generation is decreased as a result of mitochondrial dysfunction and uncoupled phosphorylation. This decrease in ATP levels act as an indirect inducer of autophagy via AMPK activation. Amino acids and free fatty acids are products from the autophagic degradation process of membrane lipid and proteins. These degradation products can be used by the tricarboxylic acid (TCA) cycle to maintain or increase mitochondrial ATP production during ischemia. This is however not an option during complete oxygen shut-down and/or severe and prolonged ischemia. In addition, some autophagic processes and autophagy itself are ATP-dependent and can therefore not occur during complete ischemia. Autophagy may therefore serve as an energy-recovering process during ischemia by compensating for the loss of energy through the "recycling" of degradation products for ATP synthesis (Levine and Yuan, 2005; Matsui *et al.*, 2008; Sciarretta *et al.*, 2011; Ma *et al.*, 2015).

The above viewpoint was confirmed by Matsui *et al*. (2007) who demonstrated that pharmacological inhibition of autophagy was responsible for a decrease in ATP production and a subsequent aggravation of cardiac cell death (Matsui *et al.*, 2007).

2. Autophagy contributes to the removal of damaged protein aggregates (proteostasis)

The two processes responsible for protein quality control include:

- Autophagy, mainly for long-lived and macromolecular proteins, and
- The ubiquitin proteasome system (UPS) for short lived proteins (Hochstrasser, 1996).

The ubiquitin proteasome pathway becomes dysfunctional during ischemia. This contributes to the accumulation of ubiquitinated proteins and resultant protein aggregates (Calise and Powell, 2013). Autophagy, on the other hand, is induced

during ischemia – and can therefore compensate for the impaired UPS function, keeping proteolysis at an appropriate level by scavenging protein aggregates that accumulated during ischemia (Levine and Yuan, 2005; Mizushima, 2007; Ma *et al.*, 2015).

3. Mitochondrial autophagy (mitophagy) mediates the removal of damaged mitochondria in the heart

Cardiac ischemia results in mitochondrial fission and fragmentation in cardiomyocytes (Hwang and Kim, 2013). Excessive mitochondrial damage will be responsible for an overproduction of reactive oxygen species with a resulting inflammatory response and potential myocardial cell death. The process by which autophagy removes these damaged mitochondria, is known as mitophagy (Quinsay *et al.*, 2010). Mitophagy is therefore another protective mechanism of autophagy during which damaged mitochondria are removed, thereby reducing the release of reactive oxygen species and inhibiting the release of pro-apoptotic factors, such as cytochrome c (Ma *et al.*, 2015).

Autophagy primarily functions as an alternative “energy source” during the initial phase of ischemia, whereas the emphasis falls on protein clearance in the later phase of ischemia and reperfusion (Ma *et al.*, 2015).

4. Inhibition of apoptosis

Autophagy triggered by ischemia can also inhibit apoptosis, and therefore reduce the deleterious effects of ischemia. This is however ischemic time sensitive, as reffered to before (Bauvy *et al.*, 2001; Yan *et al.*, 2005; Nishida, Yamaguchi and Otsu, 2008). The protective autophagic effect during chronic myocardial ischemia is therefore also contributed to by the protection against apoptosis (Yan *et al.*, 2006; Q. Xu *et al.*, 2015).

4.5.2. Support for the detrimental consequences following induction of autophagy

In contrast to the convincing protective effects of autophagy during I/R described above, there is also evidence pointing to detrimental effects associated with the upregulation of autophagy (Aki *et al.*, 2003; Valentim *et al.*, 2006; H. Zhu *et al.*, 2007; Matsui *et al.*, 2007; Lu *et al.*, 2009; Zhao *et al.*, 2010). These studies include:

- Pharmacological inhibition of autophagy (by 3MA), as well as the knockdown of Beclin 1, increased cardiac myocyte survival following ischemia and reperfusion *in vitro* (Valentim *et al.*, 2006).
- Matsui *et al*. (2007) demonstrated the protective effect of autophagic inhibition during reperfusion: in this study the infarct size averaged 20% in Beclin-1 knockout mice compared to 40% in the wild-type mice (Matsui *et al.*, 2007).
- In cells exposed to hypoxia, HIF-1α induces autophagy, enhances apoptosis and oxidative stress. 3MA (an autophagic inhibitor), in this cancer cell model, was responsible for a reduction in apoptosis and oxidative stress (Wang *et al.*, 2017).
- Aki *et al*. (2003) investigated the effect of autophagic vs apoptotic inhibition in starved (therefore autophagy induced) rat cardiomyocyte cells. This study demonstrated that cell death during starvation can be attributed to autophagic cell death and not type I programmed cell death (apoptotic cell death) (Aki *et al.*, 2003).
- 3MA was used in an adriamycin heart failure model of rats. The pharmacological inhibition of autophagy in this study lead to significantly improvement in cardiac function as well as reduced mitochondrial injury (Lu *et al.*, 2009).

<u>Potential mechanisms for the detrimental effects following autophagic induction during ischemia</u>

1. Excessively upregulated autophagy will not only remove dead cells and organelles (in an attempt to generate energy) but will be responsible for autophagy mediated cell death/autosis (Refer to section 4.2 (c) and (d)) (Kroemer and Levine, 2008; Liu *et al.*, 2013).
2. Following above, autosis is not necessarily constrained by inhibition of lysosomal breakdown and excessive autophagosome formation at the expense of intracellular membrane sources (ER) may be responsible for cellular dysfunction and death (Sciarretta *et al.*, 2018).
3. The relationship between autophagy and apoptosis during excessive upregulation of autophagy. (Refer to section 4.2 (e))

4.5.3. Factors influencing the end result of autophagic manipulation following ischemia and reperfusion

Above demonstrate that autophagy can act as a double- edged sword in the process of ischemic reperfusion injury (Cao, Hill and Gillette, 2009; Ma *et al.*, 2015). The reasons for the conflict in the literature regarding the beneficial and detrimental effects secondary to autophagy during ischemic and reperfusion injury most probable comes down to:

- The different experimental models used.
- The differences between ischemic and reperfusion, and the effect these two processes have on autophagy.
- The technique employed for the evaluation of autophagy, and whether true autophagic flux was measured.
- The degree (time period) of ischemia and reperfusion (therefore the degree of autophagic induction and the effect that it will have on autophagy and apoptosis).
- If the animals used was in a starved or in a fed state.

Matsui *et al*. (2007) alluded to the importance of the nutritional state of the study model when studying autophagy. They reported that the detrimental effect of autophagic induction during reperfusion might be because the cells are not in a starved state. This is in contrast to the ATP-depleted/starved conditions during ischemia during which the induction of autophagy might be essential for survival (Matsui *et al.*, 2007).

Another factor that might contribute to this apparently dual role that autophagy plays in the heart can be referred to as the "Goldilocks phenomenon" – just the right amount of autophagy will be beneficial but too little or too much might be detrimental to the organism (Nishida, Yamaguchi and Otsu, 2008). Low levels of autophagy (during ischemia and early reperfusion) might be protective by providing nutrition, whereas long-term upregulation of autophagy (during reperfusion) may be harmful secondary to excess degradation of essential proteins and organelles (Gustafsson and Gottlieb, 2008a). This was elegantly demonstrated in the study done by Xu *et al*. (2015). As mentioned before, their findings indicated that an enhancement of autophagy is beneficial in cardiomyocytes subjected to mild to moderate ischemia. In contrast,

during late/severe myocardial ischemia an autophagy inhibitor (3MA) protected the myocardium (Q. Xu *et al.*, 2015).

Overall, physiological levels of autophagy, presumably caused by mild to modest hypoxia, appear to be protective. On the other hand, uncontrolled excessive induction of autophagy may cause self-digestion via autophagic cell death and cellular dysfunction (Matsui *et al.*, 2007; Sadoshima, 2008; Ma *et al.*, 2015).

It may furthermore be important to distinguish between the role of autophagy during ischemia and during reperfusion, separately, and not to refer to the combined effect of "ischemic reperfusion injury". According to Matsui *et al*. (2007) an increase in autophagy may be protective during ischemia, but detrimental during reperfusion (Matsui *et al.*, 2007). In terms of reperfusion there is evidence that autophagic induction protects against early myocardial *reperfusion injury* in diabetic mice hearts, (Das *et al.*, 2015) and another study found that the effects secondary to autophagy triggered by reperfusion can be either adaptive or detrimental (Matsui *et al.*, 2007; Gatica *et al.*, 2015). These effects of autophagic manipulation during ischemia and reperfusion are most probable related to the different degrees of autophagic upregulation during these processes.

The effect of autophagic induction during reperfusion remains controversial, Hamacher-Brady *et al.* (Hamacher-Brady, Brady and Gottlieb, 2006a) have shown that enhancing autophagic flux has a protective effect, while Valentim *et al*. (2006) found the opposite (Valentim *et al.*, 2006). Another noteworthy finding is the fact that a direct correlation exists between the severity of ischemia and the degree of autophagy during the consequent reperfusion phase (Decker and Wildenthal, 1980).

Ma *et al*. (2012) supporting impaired autophagic flux during the reperfusion phase, discovered that the decrease in autophagosome clearance during reperfusion in cardiomyocytes is detrimental to cardiomyocyte survival during reperfusion (Ma, Liu, Foyil, Godar, Weinheimer and Diwan, 2012)

Based on the above, it seems that autophagy plays an important protective role in the heart under normal conditions (and therefore low levels of autophagy), but that excessive or dysregulated autophagy will be detrimental secondary to the induction of type I and II programmed cell death. The maintenance of a balance of autophagy,

even during ischemia and reperfusion, seems important – not too much and not too little (Shaw and Kirshenbaum, 2008; Chiong *et al.*, 2011)!

4.6. CONCLUSION AND MAJOR QUESTIONS ARISING FROM THE EXISTING LITERATURE

Ischemia and reperfusion induce several cellular conditions (ATP starvation, oxidative stress, endoplasmic reticulum stress, mitochondrial damage, inflammation and tissue remodeling) all of which may induce autophagy (Sadoshima, 2008).

The effect of ischemia and reperfusion, irrespective of above, on autophagy (steady state and flux) is however still a subject of debate, as are the consequences of autophagic changes secondary to ischemia and reperfusion. There is ample evidence to support protective as well as detrimental effects secondary to autophagic induction following ischemia and reperfusion.

It appears that the cardioprotective properties of autophagy during ischemia and reperfusion are context-dependent: It depends on the degree of autophagic induction and thus also the degree (severity) of the associated ischemia and reperfusion. Other reasons that might contribute to the opposing views in terms of autophagy and the effects it manipulation has on myocardial protection include the crosstalk between autophagy and apoptosis, (Sheng and Qin, 2015) the difference between true autophagy and steady state autophagy measured and the different experimental models.

What we however do know is that physiologically beneficial autophagy ought to be preserved, whereas excessive autophagy is detrimental and needs to be suppressed. The question however still remains – when does beneficial autophagy turn into detrimental autophagy? Clarification of the role of autophagy in mediating cell survival (or death) during ischemia and reperfusion, may have a significant impact on treatment of ischemia-related diseases. Modulation of this autophagic pathway could represent a future therapeutic target during ischemia and reperfusion. The relative paucity of the literature however indicates that more research needs to be done (Chiong *et al.*, 2011).

The value in doing this research is based on the fact that the heart is the organ most often affected by ischemia and reperfusion injury and that ischemic heart disease is

one of the more common health problems in the western world. The fact that reperfusion injury can contribute to up to 50% of the infarct size, in combination with the lack of existing therapeutic interventions to effectively prevent the detrimental effects of reperfusion injury, makes *reperfusion injury* an important area of research (Hausenloy and Yellon, 2013).

4.7. HYPOTHESES

Following the above review, the hypothesis for this study states that:

In the isolated perfused rat heart:

1. During *early reperfusion* autophagy induction, in contrast with inhibition, will improve global myocardial function and lead to a decrease in infarct size.
2. During *late reperfusion* autophagy inhibition, in contrast with induction, will improve global myocardial function and lead to a decrease in infarct size.

Since no similar studies have been done in the past, we base our hypothesis on literature concerning autophagy and reperfusion. We postulate that autophagy induction will be protective during early reperfusion because of the beneficial effects of controlled upregulation of autophagy. We further hypothesize that because late reperfusion (potentially) leads to an unregulated, excessive upregulation of autophagy, it causes more harm than good, therefore the inhibition of autophagy during this time period may be beneficial (Hausenloy & Yellon, 2013:92; Thapalia, Zhou & Lin, 2014:8322).

4.8. RESEARCH AIMS

The *primary aim* of this study was to investigate the protective effect of autophagy induction during early reperfusion and autophagy inhibition during late reperfusion by the use of appropriate drugs.

The *secondary aims* were to:

1. Characterize the pattern of autophagic flux during early and late reperfusion in our experimental model.
2. Investigate the effect of different periods of ischemia on autophagic flux during reperfusion.

CHAPTER 5
EXPERIMENTAL METHODOLOGY

5.1. INTRODUCTION

The purpose of this chapter is to describe the various methods and techniques used to accomplish the earlier described aims.

5.2. STUDY DESIGN

This is an interventional animal research study, during which we made use of random allocation of animals to the different groups.

5.3. ETHICS CLEARANCE AND PROTOCOL APPROVAL

Ethics approval for this project was granted by the Stellenbosch University Research Ethics Committee: Animal Care and Use via committee review procedures; Protocol #: SU-ACUD16-00061. All handling of animals complied with the accepted standards for the use of animals in research as stipulated in the South African National Standards (SANS) 10386: 2008, and by the South African Veterinary Council (SAVC) (http://www.savc.org.za).

The study strictly adhered to the principles of “replacement, reduction and refinement”. The rats were specifically bred for this research and specific numbers of rats had to be ordered well in advance. Sample size calculations (see Section 5.13) and interim analysis were done in an attempt to use the smallest number of animals possible. For each heart, protein analysis as well as assessment of global myocardial function were done.

In terms of refinement, as stated below, euthanasia (Section 5.6) was done in a way to minimize potential pain, suffering and distress. The rats were fed on a standard rat chow diet, and had free access to clean drinking water. They were housed in the University of Stellenbosch Central Research Facility, in a temperature controlled room (22°C), with 40% humidity and 12-hour artificial day-night cycle. Animal monitoring and care like daily food and water consumption, weekly total body weight measurement, cleaning of animal cages and changing of animal beddings were performed routinely.

5.4. INFRASTRUCTURE AND PERSONNEL

The cardiovascular research experiments and Western blot studies were conducted in the Division of Medical Physiology (part of the Cardiovascular Research Group), Faculty of Medicine and Health Sciences (FMHS), Stellenbosch University (SU).

Laboratory animals were bred, housed and supplied by the animal housing facility, Faculty of Medicine and Health Sciences, SU.

The following personnel were involved in the various research study activities:

- Animal monitoring and care: personnel at the animal facility, SU.
- Drug preparations: candidate (Marli Smit).
- Euthanasia, and organ harvesting: – Ms Sonia Genade (SANS and SAVC certified).
- Cardiac perfusion studies: Ms Sonia Genade and candidate.
- Lysate preparation and western blotting: candidate.
- Data management and analysis – candidate in collaboration with Biostatistics Unit, SU.
- Supervisors – Prof. A.R. Coetzee, co-supervisors Profs. Amanda Lochner and Hans Strijdom.

5.5. ANIMALS

Age and weight (210-350g) matched male, pathogen free, Wistar rats (*Rattus norvegicus*) were used in the studies. They have been extensively and successfully used in cardiovascular experimental studies in this laboratory (Lochner *et al.*, 2006). Although aware of the limitations associated with the use of male only rats, female rats were excluded in order to avoid the associated hormonal changes (Moran, Leathard and Coley, 2000). This approach contributed to limiting the sample size.

For the isolated perfused working rat hearts and subsequent western blotting, a sample size of five for each indicated time interval, for both the control and experimental groups, were used. Eight hearts per group were used for infarct size experiments.

5.6. EUTHANASIA AND HEART ISOLATION

Rats were anaesthetized through an intraperitoneal (IP) injection of sodium pentobarbitone (*Eutha-naze®, Bayer (Pty) Ltd. Animal Health Division, South Africa*) (160mg/kg body weight) using a 26-gauge hypodermic needle. The injection was aimed at the right lower quadrant of the abdomen in order to avoid injury to solid abdominal organs. Following injection, the animals were weighed and kept comfortable allowing time for the pentobarbitone to induce anesthesia. A pedal withdrawal reflex to pain stimuli was performed to confirm an adequate level of anesthesia before the surgical removal of the heart. A transverse sub-sternal incision was made to access the chest cavity, enabling cutting of the aorta and pulmonary trunk, which was followed by rapid (less than one minute) removal of the heart. After removal, the heart was submerged in fresh cold (4°C) modified Krebs-Henseleit buffer (KHB) (Table 5.1.) to arrest the heart and consequently conserve myocardial energy.

Table 5.1: Composition of Krebs-Henseleit perfusion buffer used for perfusion of the isolated rat heart (mM = millimolar)

Chemical compound	**Concentration**
NaCl	119 mM
$NaHCO_3$	24.9 mM
KCl	4.7 mM
KH_2PO_4	1.2 mM
$MgSO_4 7H_2O$	0.59 mM
Na_2SO_4	0.59 mM
$CaCl_2$	1.25 mM
Glucose	10 mM

Excess fat and connective tissue around the heart and major vessels were dissected out, after which the heart was mounted on the working heart perfusion apparatus (Neely-Morgan perfusion system) via the aortic cannula.

This was done by picking up the heart by the aorta with forceps, and slipping it onto the grooved aortic cannula. The aorta was then tightly fastened to the cannula using a cotton thread. Once cannulated, retrograde perfusion was established with KHB at

a pressure of 75 cm H_2O. The left atrium was subsequently cannulated through the pulmonary vein, to allow perfusion of the left atrium, in working heart (WH) mode, when indicated. This procedure was according to the working heart model as described by Neely *et al*. (1967) and subsequently modified by Opie and coworkers in 1971 (Neely *et al.*, 1967; Opie, Mansford and Owen, 1971).

5.7. ISOLATED WORKING HEART

Isolated rat hearts were perfused using the well-established Neely-Morgan perfusion system (Neely *et al.*, 1967). This system contained warm (36°C), freshly prepared KHB (Table 5.1) which was gassed with a mixture of 95% oxygen and 5% carbon dioxide to maintain a pH of 7.4. Before use it was filtered through a 0.45 µm mixed cellulose ester pore (Advantec MFS Inc, Pleasanton USA).

Myocardial temperature was monitored throughout the perfusion protocols with a coronary sinus placed thermistor probe. The myocardial temperature was carefully maintained at 36.5°C during (global and regional) ischemia. This was done by means of a heated water bath (Grant instruments, Cambridge, England), circulating warm water through the water- jacketed glassware. A peristaltic pump (Watson Marlow Ltd, UK) was responsible for the circulation of the buffer through the perfusion system.

After mounting of the heart on the aortic cannula, hearts were perfused in the Langendorff mode (retrograde perfusion) at 100 cm H_2O for a period of 15 min to allow cannulation of the left pulmonary vein, after which perfusion was switched to the working mode (preload 15 cm H_2O; afterload 100 cm H_2O).

Table 5.2: Exclusion criteria for terminating experiments during the stabilisation period (min = minute, bpm = beads per minute, mmHg = millilitres mercury, ml/min = millilitres per minute)

Parameter	Criteria
Time to perfusion from excision	> 3 min
Heart rate	< 200 bpm, > 400 bpm
Left ventricular developed pressure	< 85 mmHg
Arrhythmia duration	Significant cardiac arrhythmias defined as salvos, ventricular tachycardia or fibrillation occurred during the 5 min period prior to occlusion.

Coronary flow	< 10ml/min or 1ml/min or > 25 ml/min

Any criteria residing outside of these values suggested potential damage to an isolated heart during excision and cannulation and was excluded from our study (Clements-Jewery, Hearse and Curtis, 2002; Galagudza *et al.*, 2004; Norman, Cohen and Bampton, 2010; Bell, Mocanu and Yellon, 2011; Donner *et al.*, 2013).

At the predetermined times (Section 5.12) cardiac mechanical function (Section 5.9) was documented, and hearts freeze-clamped (with liquid nitrogen, pre-cooled Wollenburger tongs) and stored at -80°C for subsequent Western blot analyses (Section 5.10).

Either global or regional ischemia was induced during rat heart perfusion.

5.7.1. Global ischemia

To induce global ischemia, perfusion to the heart was completely cut off (coronary flow rate of 0 ml/min) by closing both the aortic and left atrial flows for 15 or 20 minutes, depending on the protocol.

5.7.2. Regional ischemia

Hearts that were subjected to the regional ischemia-reperfusion protocol, underwent ligation of the proximal part of the left anterior descending coronary artery (LADCA). A small silk surgical suture was used to form a snare by placing it from the midlevel of the left atrium to the midlevel of the pulmonary tract. The ligature ends were then passed through a short plastic tube to form a snare. Thirty-five minutes of regional ischemia was allowed from the moment myocardial blanching was observed. This reduced the coronary flow to approximately 30% of the pre- ischemic coronary flow rate. Shorter infarct size duration (to match the time of global ischemia of 15 and 20 min) produced infarct sizes too small for accurate measurement.

At the end of 2h of reperfusion 0.5 - 0.8 millilitres of a 0.5% Evans blue (*Sigma, St. Louis, USA*) dye suspension was slowly injected, by uniformly applied pressure, via the aortic cannula. Thereafter, hearts were frozen overnight (in a closed container at -20°C) before staining with 2,3,5 triphenyltetrazolium chloride (TTC, *Merck (Pty) Ltd. Germany*).

5.8. INFARCT SIZE ANALYSIS AND TTC STAINING

TTC staining salt solutions were prepared by mixing Solution I (20 ml) and Solution II (80 ml).

Solution I: 100 mM $NaH_2PO_4.2H_20$ (15.6 g/L dH_2O)

Solution II: 100 mM Na_2HPO_4 (14.2 g/L dH_2O)

After mixing the above solutions, TTC salt was added in a ratio of 0.05 g salt for each 5 ml. The TTC staining salt solution container was then covered with aluminium foil to protect against light.

Each frozen heart was sliced into 6-7 slices of equal (about 2 mm) thickness and then placed in a tube containing 5 ml of the above solution (0.05 g TTC /5 ml buffer solution). Thirty minutes were allowed for staining, during this time period the tube was shaken a few times to ensure even coverage. At the end of 30 minutes, the slices were removed from the TTC-buffer solution and fixed in a 10% v/v formaldehyde solution, this was left to stand for 90 min at room temperature. The formaldehyde enhanced the contrast between the stained and unstained areas. Colour interpretation was as follows: Dark/blue area represents viable and undamaged tissue; the white/unstained area indicates dead (infarcted) tissue and the combination of the white and red areas indicated the area at risk. (Figure 5.1)

Directly following staining the slices were placed between glass plates and redrawn, copying the colours and sizes of the respective stained areas. The images were then scanned for computerized planimetry. Quantification was done using an image analysis software (Image Tool, *University of Texas, Health Science Centre, San Antonio Texas, UTHSCSA,* http://ddsdx.uthscsa.edu/dig/itdesc.html). The infarct size of the left ventricle was expressed as a percentage of the area at risk.

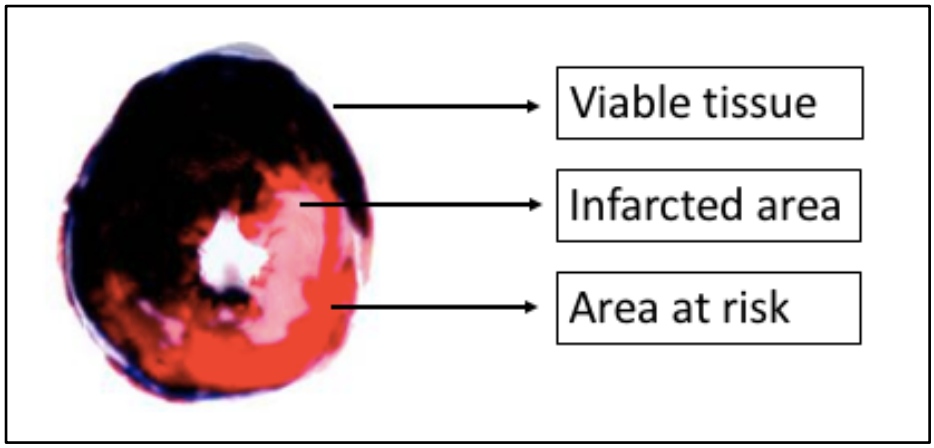


Figure 5.1: Transverse section of the rat heart, post TTC staining

Infarct size (IS) = Infarct area / (Area at risk + Infarct area) x 100. The ischemic area include the infarcted area (white) and the area at risk (red).

5.9. CARDIAC HEMODYNAMIC PERFORMANCE DATA

Functional parameters monitored and collected include systolic and diastolic intra-aortic pressure, heart rate (HR), coronary (Qe) and aortic (Qa) flow rates. Coronary and aortic flow rates in millilitre/minute (ml/min) were manually measured by timed collection of the perfusate, and used to calculate the total cardiac output (CO). CO = Qe + Qa

The intra-aortic pressure (in mmHg) and heart rate (in beats per minute, bpm) were measured with a pressure transducer inserted into a side branch of the aortic cannula.

Functional recovery of the hearts was determined by comparing post-ischemic CO and total work (Wt) with pre-ischemic CO and Wt. The left ventricular work performance was calculated as described by Kannengiesser (Kannengiesser, Opie and der Werff, 1979).

Total work = $0.002222\ (P_{AO} - 11.25)(CO)$ where,

P_{AO} = aortic pressure and CO = cardiac output

5.10. MEASUREMENT OF AUTOPHAGY: WESTERN BLOTTING

Different options, as discussed in Section 1.6, page 15, to measure autophagy include electron microscopy, (Iwai-Kanai *et al.*, 2008) fluorescence microscopy (Matsui *et al.*, 2007) and biochemical assays. The most common assays include detection of Beclin-

1, p62/SQSTM1, LC3-II and the LC3-II/LC3-I ratio by immunoblotting of tissue lysates (Mizushima, Yoshimori and Levine, 2010).

We made use of the above-mentioned proteins as well as markers of an alternative pathway namely (phosphorylated and total) ULK1, (phosphorylated and total) DRP1 and Rab9 to measure steady state autophagy as well as autophagic flux. Intra-peritoneal administered Chloroquine, (Hong *et al.*, 2004) 1h before experimentation, was used to determine flux (Section 7.5.3).

The freeze-clamped hearts (Section 5.12) were used for protein identification and quantification by means of western blotting, which consist of the following steps.

5.10.1. Lysate preparation and tissue homogenization

All steps for lysate preparation were done on ice. From each frozen heart, 0.15 to 0.2 g was weighed and pulverized using a liquid nitrogen-precooled mortar and pestle. The pulverized powder was added to 700 µL of lysis buffer (see below) to which eight 1,6 mm and three 2 mm stainless-steel beads (*Next Advance, New York, USA*) were added and then homogenized using the bullet blender^{TM} (*Next advance, New York, USA*) at 4°C. The bullet blender was set at homogenization power of ten for two two-minute cycles, allowing a one-minute rest interval between the cycles. After a rest period of 15 min, the samples were centrifuged (at a temperature of 4°C) for 20 minutes at 15,000 rpm (*Sigma Laborzentrifugen,* Osterode am Harz, *type 1-4 K, Germany*). Following centrifugation, 1000 µL of each sample's supernatant was transferred to an Eppendorf tube for the Bradford protein assay and subsequent storage.

The lysis buffer referred to above contained: 20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 1 mM β-glycerophosphate, 2.5 mM tetrasodium pyrophosphate, 1 mM sodium orthovanadate, 1% Triton X-100, 10 µg / mL leupeptin, 10 µg / mL aprotinin and 50 µg / mL PMSF.

Table 5.3: Summary of protein (primary antibody) information for western blotting

Protein	MW kDa	Gradient gel	Protein loaded per 12 ul load	Primary AB dilution factor	Secondary AB dilution Factor	Duration of ECL incubation prior to exposure	Exposure time in Chemi Doc (seconds)
SQSTM1/ p62	60	4-20%	30ug	1: 1000	1 : 4000	3 min	200
LC3A/B	14 , 16	4-20%	30ug	1: 1000	1: 4000	30 sec	30
Beclin-1	60	4-20%	30ug	1 :1000	1: 4000	5 min	200
Rab9	23	4-20%	30ug	1 :1000	1: 4000	1 min	100
ULK 1	150	4-20%	30ug	1 :1000	1: 4000	4 min	180
Phospho ULK 1	140-150	4-20%	30ug	1 :1000	1: 4000	4 min	500
DRP1	78–82	4-20%	30ug	1 :1000	1: 4000	30 sec	30
Phospo DRP1	78-82	4-20%	30ug	1 :1000	1: 4000	3 min	430
Caspase	35	4-20%	30ug	1 :1000	1: 4000	3 min	210

Abbreviations: AB: antibody, min: minute, kDA: kilodalton

5.10.2. Bradford protein assay (BPA)

The protein concentration of the supernatant was determined by using the standard Bradford method (Bradford, 1976).

Bradford stock was prepared by dissolving 500 mg coomassie brilliant blue in 250 ml 95% ethanol. Thereafter 500 ml of phosphoric acid was added, followed by distilled water to make up a final volume of 1 L. From this stock, Bradford solution was prepared by diluting it 1:5 with distilled water and filtered twice using Whatman filter papers (0.4 µm pores). Into sorval tubes 10 µL of each sample supernatant was added to 90 µL of distilled water. From this 10-time diluted set, a 5 µL volume was drawn (for each sample) and added to another set of duplicate tubes with 95 µL of distilled water.

To prepare a standard curve, 100 µL Bovine serum albumin (BSA) of known concentration (5 mg/ ml) was diluted five times (400 µL of distilled water was added). This working standard was used to prepare a standard curve as depicted in Figure 5.2.

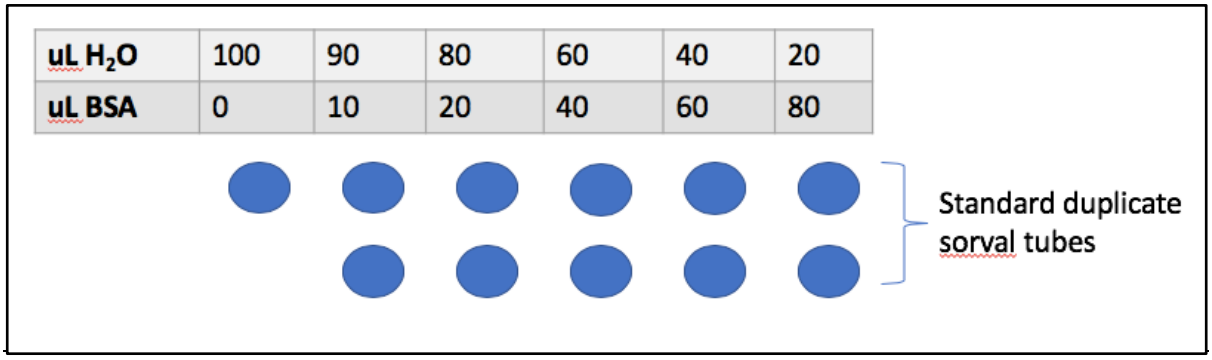


Figure 5.2: Graphic representation for the standard dilutions

They were made up with increasing concentrations of Bovine serum albumin (BSA) and decreasing volumes of distilled water (H2O). It was done in duplicate, as depicted.

Following this, 900 µL of the filtered Bradford solution was first added to the standard duplicates and then to the diluted samples. Samples were analysed in duplicate. All samples were thoroughly vortexed and incubated for 15 minutes on the bench to allow for colour development, after which the absorbance was measured using a spectrophotometer (Cat. 4001/4, *Spectronic Instruments, USA*).

Using the absorbance values of the standard BSA curve, protein concentrations of the samples were calculated using the linear equation y = mx + c.

The final lysates consisted of supernatant, Laemmli buffer and lysis buffer. Based on the above determined protein concentrations of each sample, the pre-calculated volume of

supernatant was used, aiming for a final lysate protein concentration of 30 µg per 12 µL volume. One third of the volume of lysates was composed of the Laemmli sample buffer (62.5 mM Tris-HCl (pH 6.8), 4% SDS and 10% glycerol, 0.03% bromophenol blue and 5% β-mercaptoethanol). The volume difference (12 µL minus the supernatant and the Laemmli buffer) was topped up with the lysis buffer. The prepared lysate samples were boiled in water (at 100°C) for five minutes in order to denature proteins, and stored at -80°C.

5.10.3. Protein separation and gel to membrane transfer

Before the lysates were loaded in the SDS-polyacrylamide gels to separate the proteins in terms of size, it was once again incubated in boiling water (100°C) for five minutes to denature proteins. Equal amounts of protein (30 ug) were loaded and separated using Criterion™ TGX Stain-Free™ precast gradient (4-20%) gels (Bio-Rad). The protein marker used was the PageRuler™ Prestained Protein Ladder (Thermo Scientific). The running buffer used during protein separation contained the following: 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS. The gels were electrophoresed at 200 mA and 200 V for 55 minutes using the Midi Trans-Blot® Cell system from Bio-Rad, after which the gels were Stain-Free activated for one minute and imaged using the ChemiDoc MP system and ImageLab software 5.2.1 (Bio-Rad).

After separation and gel activation, polyvinylidene fluoride membranes (PVDF, Immobilon®-P, Millipore) were immersed into methanol after which the proteins were transferred onto the PVDF membrane using the Trans-Blot® Turbo ™ Transfer (semi-dry) System and RTA Transfer Kits from Bio-Rad. Use was made of the mixed molecular weight (5-150 kD) program (7 minutes duration at 2,5 A and a voltage of up to 25 V) with the transfer buffer containing 100 ml of 5X transfer buffer, 300 ml nanopure water and 100 ml ethanol. After the transfer was completed, the membranes were imaged using the ChemiDoc MP system and ImageLab software 5.0 (Bio- Rad) in order to do whole lane protein normalization to correct for any unequal loading.

5.10.4. Membrane blocking, primary and secondary antibody incubation and visualisation of the proteins

Non-specific binding sites were blocked for two hours using 5% (w/v) fat-free milk made up in Tris-buffered saline-Tween-20 (TBST) buffer (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Tween-20). Following blocking, the membranes were washed thoroughly with TBST

for three cycles of 10 minutes on a shaker and incubated overnight (± 16 hours) under constant agitation with the appropriate primary antibody at 4°C. Primary antibodies used are summarised in Table 5.2. All primary antibodies were raised in rabbit and acquired from Cell Signaling Technology.

The following day the membranes were washed (for 30 minutes with TBST) to remove any unbound primary antibody and incubated with anti-rabbit immunoglobulin G, Horseradish peroxidase-linked (from donkey) secondary antibody (Cell Signaling Technology) at room temperature (± 23°C) for one hour. During this hour, the conjugated antibody had the opportunity to bind to the already bound primary antibody. Following this, the unbound secondary antibody was washed away using TBST (three cycles of 10 minutes).

In order to visualise the proteins of interest, a chemiluminescent detection system was employed. The membranes were incubated in Clarity^{TM} Western ECL Blotting Substrate (Bio- Rad) for different time periods (Table 5.2) after which the membranes were exposed using the “Chemi” or “Chemi High Sensitivity” (for p-ULK, p-DRP and T-ULK) settings of the ChemiDoc MP system (Bio-Rad). The ECL reacts with the Horseradish peroxidase (that is linked to the secondary antibody) in a luminescence reaction and the resulting light emission is visualized.

The primary antibodies were made up in a concentration of 1:1000, diluted with TBST solution or signal boost (for T-ULK, p-ULK and p-DRP). The secondary antibodies were made up to a concentration of 1:4000, diluted the same as for the primary antibodies.

Periodically the PVDF membranes were stripped to enable probing for the phosphorylated proteins. The following protocol was used: Two five minutes washing cycles in distilled water, followed by seven minutes in 0.2 M NaOH, and two five minutes washing cycles in distilled water again. All steps relating to stripping took place on a shaker with gentle agitation at room temperature (± 23°C).

5.10.5. Normalisation and analysis

The ImageLab Software (version 5.0) was used to quantify and analyse the bands of interest. Signal intensities (using ECL) of the relevant protein bands as well as total protein stain-free fluorescence were determined using the “Lane and Bands” function of the above-mentioned software.

In order to correct for any unequal loading and/or process inconsistencies, the blots were

normalised against the total protein in a specific lane using the “Normalization” function of the software. The “Normalization” function compensates for the non-sample-related variations in protein load. This is done by determining the total protein present in each lane, which is used to calculate the normalization factor of each lane relative to the first (control) lane. A lane with less protein loaded/transferred will have a higher normalization factor and a lane with more protein loaded/transferred will have a lower normalization factor (Gürtler *et al.*, 2013).

For comparison purposes, a sample from a control heart (exposed to only 10 minutes perfusion) was included in each blot and used for normalization of the treated samples. Normalized data was expressed in arbitrary units (AU).

5.11. DRUGS USED

5.11.1. Rapamycin

Rapamycin (Sirolimus) is a natural product and a lipophilic macrolide antibiotic, known for its potent antifungal and immunosuppressive activities (Kim *et al.*, 2002). It is well-known for, and often used to enhance autophagy by inhibiting the mammalian target of rapamycin (mTOR) (Hartford and Ratain, 2007; Tanemura *et al.*, 2012). Although autophagy is theoretically being induced by starvation and inhibited by nutrient rich conditions, Noda *et al*. discovered that Rapamycin can induce autophagy even in nutrient rich conditions (Noda and Ohsumi, 1998).

The mTOR pathway consist of two functional complexes: mTOR complex one (mTORC1) and mTOR complex two (mTORC2) (Sarbassov, Ali and Sabatini, 2005).

Rapamycin does not influence both mTOR complexes, and only interacts with one mTOR complex via the FK506-binding protein 12 (FKBP12) (Sabatini *et al.*, 1994). The Rapamycin sensitive mTOR complex (mTORC1) consist of mTOR, raptor (regulatory associated protein of mTOR) and GβL (G-protein β-subunit like protein). The rapamycin-insensitive complex (mTORC2) also consist of mTOR and GβL, but, instead of raptor, a protein called rictor (Sarbassov, Ali and Sabatini, 2005). It appears that Rapamycin’s mechanism of action involves the destabilization of raptor–mTOR interaction (Kim *et al.*, 2002; Sarbassov, Ali and Sabatini, 2005; Sarkar *et al.*, 2005).

The specific dosage, method of administration and the duration of exposure (Woodrum, Nobil and Dabora, 2010) of Rapamycin in the rat heart varies a lot in the literature (Table

7.10), and will be discussed in detail in Chapter 7.

We initially used 1 nM which didn't upregulate autophagy, repeated experimentation with 100 nM also resulted in disappointing results. Thereafter we increased the dosage to 250 nM, we also prolonged the duration of exposure from 10 minutes to 30 minutes, all in an attempt to effectively induce autophagy (also refer to Section 5.12.3).

5.11.2. 3 Methyl-adenine

3 Methyl-adenine (3MA) (used at 2,5 mM) is a selective inhibitor of class III phoshoinositol-3-kinase (PI3K) and inhibits autophagy by blocking the recruitment of lipid to the autophagosome, and therefore interferes with the formation of autophagosome (Hou *et al.*, 2012; Y. Yang *et al.*, 2013). It is currently the most widely autophagy inhibitor used (Seglen and Gordon, 1982; Y. Yang *et al.*, 2013).

Rapamycin and 3MA were administered via a side-arm directly into the aortic cannula; all drugs were administered in the Langendorff mode and in a re-circulating manner. 3MA (and the original 1 nM Rapamycin protocol) were re-circulated for 10 min; during the revised 250 nM Rapamycin protocol, freshly made Rapamycin was recirculated twice for 15 min periods, as discussed in section 5.12.

5.11.3. Chloroquine

Chloroquine (at 10 mg/kg), as mentioned, was employed to measure autophagic flux. It is responsible for blocking autophagosome-lysosome fusion (Poole and Ohkuma, 1981; Kawai *et al.*, 2007) and consequently the inhibition of the degradation phase of autophagy (Iwai-Kanai *et al.*, 2008). Chloroquine was initially developed to be used as an antimalarial drug. In many areas, the Plasmodium parasites responsible for malaria have, however, become resistant to chloroquine treatment (Sidhu, Verdier-Pinard and Fidock, 2002). Other uses for chloroquine have also emerged which include the treatment of rheumatic diseases and for the treatment of viral infections (Savarino *et al.*, 2003). Long term use of chloroquine has, however, been shown to have toxic effects, particularly in the eyes and heart (Fragasso *et al.*, 2009; Michaelides *et al.*, 2011).

5.12. EXPERIMENTAL PROTOCOL

After the heart was mounted, it was subjected to a predetermined perfusion protocol, as described below.

5.12.1. Stabilization

During stabilization, the heart was perfused for 15 min in the Langendorff (otherwise known as retrograde) perfusion mode, after which the flow was changed to 15 min antegrade perfusion, also known as working heart (WH) mode.

During this period, we assessed the autophagy marker LC3 (Microtubule-associated proteins light chain 3) at 10, 20 and 30 minutes. This was to confirm that autophagy remained stable throughout this period.

5.12.2. Control experiments

Control experiments were conducted in an attempt to determine the most appropriate global ischemic and reperfusion times to allow for pharmacological autophagic manipulation during the interventional experiments. Longer global ischemic as well as reperfusion times would theoretically allow better manipulation. The hearts were initially subjected to 25 min of global ischemia (n=6), but none of these hearts were able to produce aortic output during the last 10 min of working heart as set out in the protocol (Figure 5.6). Aiming to avoid the 5-10% decrease per hour in contractile function from influencing our results, 120 min reperfusion was elected as the longest reliable reperfusion period.

After 30 minutes stabilization, the hearts were thus randomly subjected to 15 *or* 20 minutes of normothermic zero-flow *global* ischemia at 36.5^{o}C followed by a total of 120 minutes reperfusion. The control experiments consisted of seven different time intervals (after which the heart was freeze-clamped) for *each* ischemic group. The groups were divided up as follows:

At the end of stabilization (**1**), at the end of ischemia (**2**), after 10 and 30 minutes of reperfusion (early reperfusion) (**3&4**) (Kitakaze, Weisfeldt and Marban, 1988) and after 60, 90 and 120 minutes of reperfusion (late reperfusion) (**5,6&7**) (Hale and Kloner, 1988; Mocanu *et al.*, 2000; Duchatellier, Brown and Alhaddad, 2001).

The amount of steady state autophagy, autophagic flux as well as hemodynamic data for functional myocardial recovery were measured in *both* ischemic groups after each of the seven intervals. (Refer to Figure 5.3)

To determine autophagic flux, Chloroquine was administered 1 hour before experimentation, after which all (of the above) control experiments were repeated (Zhang, Yao, Fang, Zhou, Gong & Li, 2014:758).

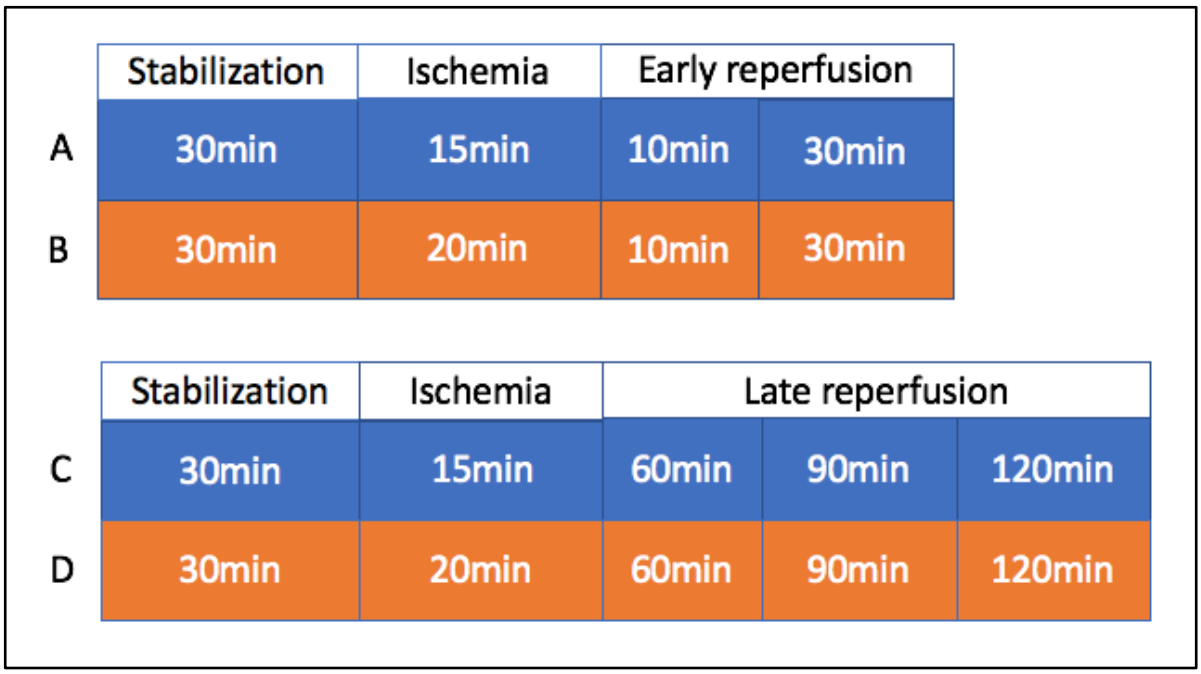


Fig 5.3: Diagrammatic presentation of the four control groups

Group A – early reperfusion after 15 min global ischemia. Group B – early reperfusion after 20 min global ischemia. Group C – late reperfusion after 15 min global ischemia. Group D – late reperfusion after 20 min global ischemia. All four groups were repeated with administering Chloroquine 1 hour before experimentation. Hemodynamic measurements for functional myocardial activity were registered and hearts freeze-clamped (for autophagic measurement) at the end of each time interval. Abbreviations: minutes: min.

To determine infarct size (for the control group) the same protocol was employed with induction of 35 minutes of *regional* (not global) ischemia. The infarct size was measured at the end of 120 min of reperfusion only.

5.12.3. Interventional experiments

The control experiments (above) were employed to determine baseline values as well as the most appropriate ischemic and (early and late) reperfusion time periods for the experimental part of our study.

Following results obtained from the control experiments the following time intervals for the interventional experiments were selected:

- 20 min for global ischemia,
- 30 min reperfusion for the early reperfusion group, and
- 120 min reperfusion for the late reperfusion group.

The ischemic time is important considering that there is a direct association between the severity of ischemia and the amount of autophagy during the reperfusion phase (Sciarretta *et al.*, 2011). The decision for the most desirable ischemic, early and late reperfusion times

was based on the time period which would allow for (potentially) the most pronounced manipulation of autophagy and myocardial protection.

In the *early reperfusion group*, Rapamycin or 3 Methyl-adenine was administered for the first 10 min following ischemia. After completion of early reperfusion (30 min) the levels of autophagy as well as myocardial function were measured in both (Rapamycin and 3MA) groups. In the *late reperfusion group* Rapamycin and 3 Methyl-adenine were infused after 50 min of reperfusion for a period of 10 min. Autophagy and myocardial function were measured after completion of the late reperfusion interval (120 min) (Figure 5.4).

Assessement of autophagic flux was performed in both the early and late reperfusion groups (for Rapamycin and 3MA), by IP administration of Chloroquine 1 hour before heart isolation.

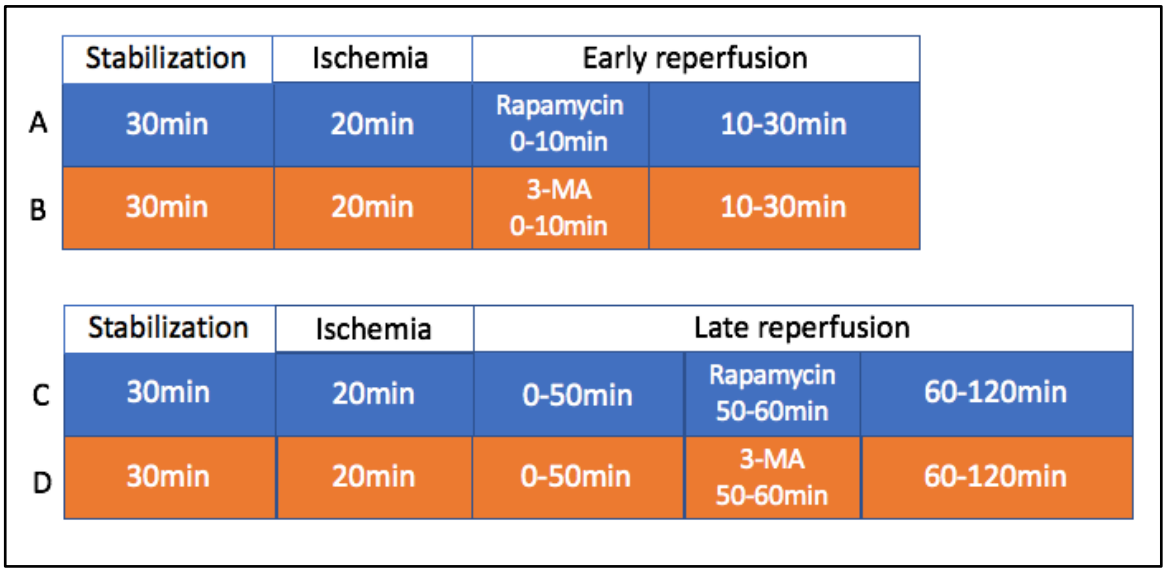


Fig 5.4: Diagrammatic presentation of the four experimental groups for global ischemia

Group A – Early reperfusion with Rapamycin. Group B – Early reperfusion with 3MA. Group C – Late reperfusion with Rapamycin. Group D – Late reperfusion with 3MA. All four groups were repeated with administering Chloroquine 1 hour before the experiment. Hemodynamic measurements were registered and hearts freeze-clamped for autophagic measurement at the end of the reperfusion period (30 min for early reperfusion and 120 min for late reperfusion). Abbreviations: min: minutes, 3MA: 3 Methyl-adenine.

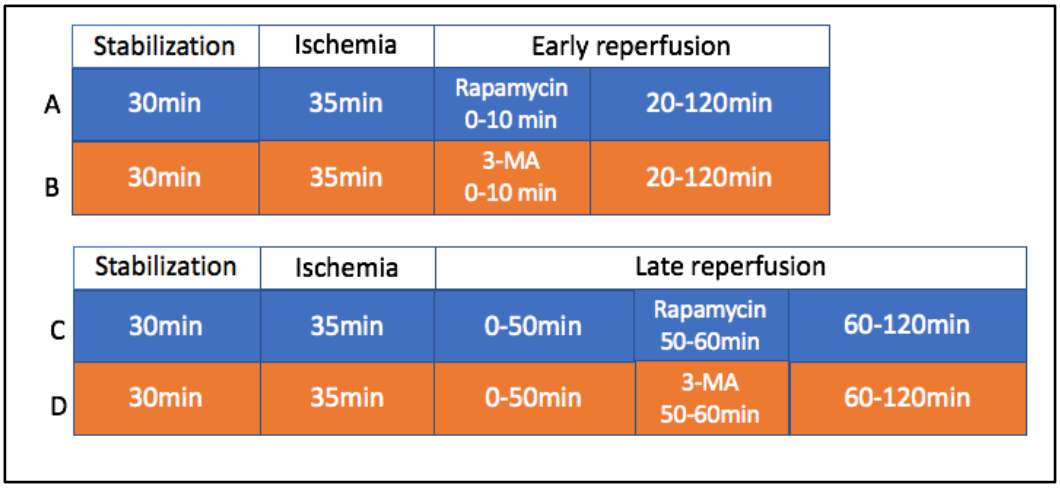


Fig 5.5: Diagrammatic presentation of the four experimental groups for regional ischemia

Group A – Rapamycin administration during early reperfusion. Group B – 3- Methyl adenine administration during early reperfusion. Group C – Rapamycin administration during late reperfusion. Group D – 3-Methyl adenine administration during late reperfusion. All four groups were repeated with administering Chloroquine 1 hour before experimentation. Hearts were prepared for infarct size measurement at 120 min reperfusion for both the early and late reperfusion group. Abbreviation: See Fig 5.2.

The exact protocol, in terms of the type of perfusions (Langendorff or work heart) used, are depicted in Figure 5.6. In the **early reperfusion, global ischemia group,** stabilization (15 min Langendorff mode, 15 min WH) was followed by 20 min of global ischemia. Reperfusion after global ischemia consisted of 10 min Langendorff perfusion (drugs were administered during this phase), followed by 20 min WH perfusion.

In the **late reperfusion, global ischemia group,** stabilization was also followed by 20 min of global ischemia. The first 10 min of reperfusion was in the Langendorff mode, the next 20 min (10-30 min) were in working heart mode, 30-60 min Langendorff mode (last 10 min used for drug administration). The first 10 minutes following drug administration (60-70 min reperfusion) was again in working heart mode and the rest of reperfusion (70-120 min) was in Langendorff mode.

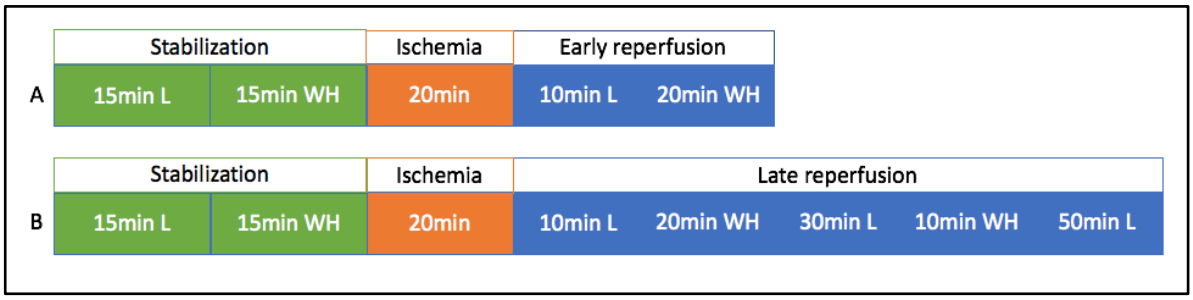


Fig 5.6: Diagrammatic presentation of the exact protocol in terms of the type of perfusion of the hearts during early and late perfusion

Group A – Early reperfusion protocol. Group B – Late reperfusion protocol. Abbreviations: WH: working heart; L: Langendorff mode; min: minute.

For the **early and late reperfusion, regional ischemia groups**, the same protocol was followed except for the fact that the *regiona*l ischemia lasted for 35 min. In these groups, which were used for infarct size determination, all the hearts were subjected to 120 min of reperfusion (essential for the tetrazolium method). The drug delivery was however during the same (early (0-10 min) or late (50-60 min)) reperfusion time intervals.

We did not get the desired results (an increase in autophagy) with Rapamycin, an autophagy inducer. We therefore repeated the experiments, with a 100 times increase in Rapamycin dosages, still without success. Thereafter the dosage was increased 250 times and the exposure time increased from 10 min to 30 min. To limit the detrimental effects of administering drugs in a re-circulatory manner (exposure to the by- and waste-products of metabolism) for 30 min (in comparison to the original 10 min), we re-circulated freshly prepared Rapamycin for 15 min in two consecutive intervals.

Since drug administration can only occur during Langendorff perfusion, the protocol was changed to allow for the increased drug exposure time. This new protocol (Figure 5.7) was employed to repeat the control experiments, as well as interventional experiments for Rapamycin with and without Chloroquine. Early reperfusion was still defined as 30 min with Rapamycin now being administered throughout this period. Late reperfusion still lasted 120 min, and drug administration took place from 50 – 80 min of reperfusion.

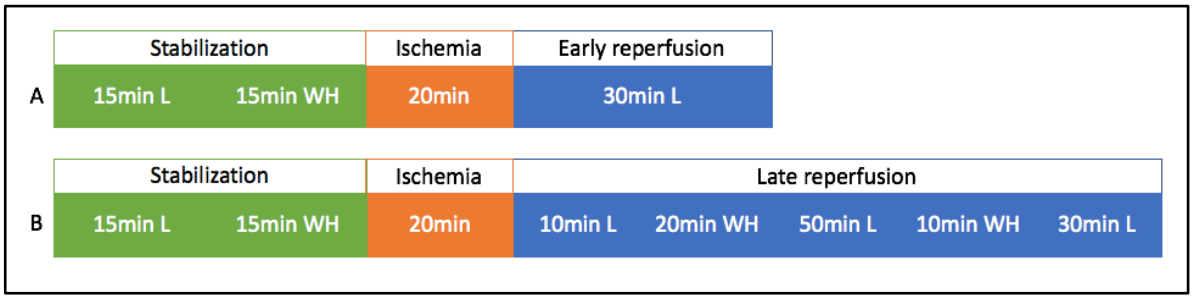


Fig 5.7: Diagrammatic presentation of the revised protocol (to allow extended Rapamycin administration) in terms of the type of perfusion of the hearts during early and late perfusion

Group A – Early reperfusion protocol. Group B – Late reperfusion protocol. Abbreviations: see Figs 5.1, 5.4.

5.13. DATA MANAGEMENT AND STATISTICAL ANALYSIS

The assistance of a Biostatistician, Dr Birhanu Ayele, from the Biostatistics Unit of Stellenbosch University (Faculty of Medical and Health Sciences) was sought.

GraphPad Prism (GraphPad Prism® Plus Version 7.0 and 8.0) was used to perform statistical analysis. All data were presented as mean ± standard error of the mean (SEM), unless otherwise stated. When comparing two groups, an unpaired Student's t-test was utilised. For multiple, normally distributed data group comparisons, one-way analysis of variance (ANOVA) with a Bonferroni post-hoc test (for differences between selected groups) was used. A p value of less than 0,05, will be indicated with *, and was considered to be statistically significant in terms of western blotting results, change in the infarct size as well as global myocardial function.

In any study, estimation of the required sample size is crucial and it depends on the variability of the population amongst other things. A too small sample size can miss the real effect in an experiment even if it exists in the target population, while a sample size that is larger than necessary will lead to unnecessary wasting of resources and ethical issues on the sacrificed animals. However, sample size calculations in animals is different from sample size for human studies. In humans, we expect a great degree of inter-individual variability (for which the sample size will compensate), while in animal studies the subjects are usually homogenous. We made use of age and gender matched rats to limit the variability between subjects as much as possible. With a homogenous group like the one we used, the result is not expected to change significantly with change in sample size (as would be the case in human studies).

We also applied strict quality control measures to compensate for measurement error. To compensate for measurement error, minor inter-individual subject differences use of the "resource equation" method for sample size calculation in combination with similar previous cardiovascular research using rat hearts (Festing and Altman, 2002; Festing, 2006; Charan and Kantharia, 2013; Arifin and Zahiruddin, 2017).

Based on this approach, the acceptable range of degrees of freedom (DF) for the error term in an analysis of variance (ANOVA) is between 10 to 20 (Festing and Altman, 2002; Festing, 2006). If it is less than 10 then adding more animals will increase the chance of getting more significant result, but if it is more than 20 then adding more animals will not increase the chance of getting significant results. Any sample size, which keeps DF between 10 and 20 should be considered as an adequate.

For one-way ANOVA, the between-subject error DF (that is, the within-subject DF) is calculated as: DF = $N - k = kn - k = k(n - 1)$,

where *N* = total number of subjects, *k* = number of groups, and *n* = number of subjects per group. By rearranging the formula, *n* is given as: *n* = DF/*k* + 1

Based on the acceptable range of the DF, the DF in the formulas are replaced with the minimum (10) and maximum (20) DFs to obtain the minimum and maximum numbers of animals per group (Charan and Kantharia, 2013; Arifin and Zahiruddin, 2017):

Minimum *n* = 10/*k* + 1 and Maximum *n* = 20/*k* + 1

Western blot, Control studies:

Early reperfusion (PI, 10'R, 30'R with and without CQ)

Minimum n = 10/6 + 1 = 2.6

Maximum n = 20/6 + 1 = 4.3

Late reperfusion (PI, 60'R, 90'R, 120'R with and without CQ)

Minimum n = 10/8 + 1 = 2.25

Maximum n = 20/8 + 1 = 3.5

Western blot, Interventional studies: (Rapa and 3MA, with and without CQ)

Early and late reperfusion:

Minimum n = 10/4 + 1 = 3.5

Maximum n = 20/4 + 1 = 6

This equates to between 3-6 hearts per group, we elected to perfuse 5 hearts per group as per the current requirement in literature as well as other similar studies (Hausenloy, Mocanu and Yellon, 2004; Lochner *et al.*, 2006, 2009; Loos *et al.*, 2011; Charan and Kantharia, 2013; Nduhirabandi *et al.*, 2017).

Work heart data: Specific sample size calculations were not done for the measurement of global myocardial function since we were required to do perfusions of all the reperfusion groups (10, 30, 60, 90 and 120 min) for blot purposes. Work heart data was documented (in these hearts) in all the different time intervals. Work heart data measurements were taken at pre-ischemic, 30 and 70 min reperfusion intervals. We therefore effectively had too many

data points which could lead to statistical significance without any clinical significance. We therefore based our sample sizes (5-10 per group) for work heart data on previously done studies (Wu *et al.*, 2013; Smith *et al.*, 2016).

Infarct size analysis:

Early and Late reperfusion: (C, C+CQ, 3MA, 3MA+CQ, Rapa, Rapa+CQ)

Minimum n = 10/6 + 1 = 2.6

Maximum n = 20/6 + 1 = 4.3

Keeping the high variability of infarct size measurement in mind as well as previous similar studies (Yang *et al.*, 2010; Wu *et al.*, 2013) we decided to study 6-8 hearts per group for infarct size analysis.

CHAPTER 6
DATA MANAGEMENT AND STATISTICAL ANALYSIS

6.1. INTRODUCTION

Following the control and interventional protocols described in Chapter 5, the findings of this study will be reported based on the different ischemic and reperfusion intervals as well as the drugs administered; as outlined in Figure 6.1.

Layout of results

Control experiments

Without CQ						With CQ					
15min GI			20min GI			15min GI			20min GI		
Early R			Late R			Early R			Late R		
Pre-I	10R	30R	60R	90R	120R	Pre-I	10R	30R	60R	90R	120R
Cardiac functional parameters (Qc, Qa, CO, PSP, HR and Wtot) were measured at 30 min for early R and 70 min for late R in all four groups. Autophagic markers (LC3A/B, Beclin and P62) were measured at the end (i.e. after pre-I, 30 min R and 120 min R) of all 24 time intervals above.											

Without CQ	With CQ
35min RI	35min RI
Infarct size measured (at the end of 120 min reperfusion) in the groups above, following 35 min of regional ischemia (RI).	

Interventional experiments

Without CQ				With CQ			
Rapamycin 1nM		3MA		Rapamycin 1nM		3MA	
Early, 30R	Late, 120R	Early, 30R	Late, 120R	Early, 30R	Late, 120R	Early, 30R	Late, 120R
Cardiac functional parameters (Qc, Qa, CO, PSP, HR and Wtot) were measured at 30min for early R and 70min for late R and Autophagic markers (LC3A/B, Beclin, P62, T and P ULK1, T and P DRP1 and Rab9) measured at the end (i.e. after 30min R and 120min R) of the 8 above mentioned time intervals.							

Without CQ				With CQ			
Rapamycin 1nM		3MA		Rapamycin 1nM		3MA	
Early, 120R	Late, 120R	Early, 120R	Late, 120R	Early, 120R	Late, 120R	Early, 120R	Late, 120R
Infarct size measured in 8 groups above. Early and late refers to drug administration, all infarcts were done at 120 min reperfusion, following 35min of RI.							

<table>
<tr><th colspan="4">Additional interventional experiments</th></tr>
<tr><th colspan="2">Without CQ</th><th colspan="2">With CQ</th></tr>
<tr><td colspan="2">Rapamycin 100nM</td><td colspan="2" rowspan="2"></td></tr>
<tr><td>Early, 30R</td><td>Late, 120R</td></tr>
<tr><td colspan="2">Autophagic markers (LC3A/B, Beclin, P62, T and P ULKI-1, T and Rab9) measured in both groups above.</td><td colspan="2"></td></tr>
<tr><td colspan="2">Rapamycin 250nM with extended exposure</td><td colspan="2">Rapamycin 250nM with extended exposure</td></tr>
<tr><td>Early, 30R</td><td>Late, 120R</td><td>Early, 30R</td><td>Late, 120R</td></tr>
<tr><td colspan="4">Cardiac functional parameters (Qc, Qa, CO, PSP, HR and Wtot) were measured at 30min for early R and 70min for late R and
Autophagic markers (LC3A/B, Beclin, P62, T and P ULK1, T and P DRP1 and Rab9) were measured at the end (i.e. after 30min R and 120min R) of the 4 groups above.</td></tr>
<tr><td>Early, 120R</td><td>Late, 120R</td><td>Early, 120R</td><td>Late, 120R</td></tr>
<tr><td colspan="4">Infarct size measured in the 4 groups above. Early and late refers to drug administration, all infarcts were done at 120 min reperfusion. (Because of the change in protocol (section 5.12), new control infarct sizes were also done for this group.)</td></tr>
</table>

Figure 6.1: Graphic representation of the layout of the control and interventional experiments

Drugs were administered from 0-10 min reperfusion in the early reperfusion group and from 50-60 min in the late reperfusion group. In the extended exposure Rapamycin group administration occurred from 0-30 min and from 50-80 min in the early and late reperfusion groups respectively. The global ischemic times were 15 or 20 min in the control groups (as indicated) and 20 min in all the interventional experiments. Regional ischemic duration was 35 min for infarct size analysis. Abbreviations: CQ: Chloroquine, GI: global ischemia, RI: regional ischemia, pre-I: pre-ischemia, R: reperfusion, min: minutes, 3MA: 3 Methyl-adenine, Qc: coronary flow, Qa: aorta flow, CO: cardiac output, PSP: peak systolic pressure and Wtot: total work.

6.2. ISOLATED WORKING RAT HEART PERFUSION DATA

The mean and SEM of the heart rate (HR in beats per minute), coronary flow rate (Qc, in mL / min), aortic output (Qa, in mL / min), total cardiac output, (CO in mL / min), peak systolic pressure, (PSP in mmHg) and total work (Wtot in mWatts), recorded during the control, control plus Chloroquine and interventional experiments (with and without Chloroquine), following global ischemia and different drug combinations, are summarised and can be found, with the rest of the perfusion data, in Appendix A.

Graphic representation of the most relevant group comparisons are illustrated and will indicate significant differences (* $p < 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p<0.0001$) between groups. The n value averaged 5-10 in all the groups. On the graphs, the x axis are used to indicate the different groups, while the y axis is labelled “arbitrary units”. The latter is because the units for CO, Wtot and Qc are not similar, the exact values on the y axis are however correct. If no reference is made to differences then there was no statistical difference.

6.2.1. Control experiments: 15 min and 20 min of global ischemia

As expected, following the control experiments, a statistical significant decrease in **CO** was found following 15 min of global ischemia. This decrease was observed at 30 min (41.40±2.08 ml/min, $p<0.0001$) and 70 min (35.10±1.66 ml/min, $p<0.0001$) reperfusion, when compared to the pre-ischemic (58.15± 1.96 ml/min) period (Figure 6.2). A significant reduction also occurred after 20 min of global ischemia, when comparing the CO during the Pre-I interval (51.25±1.28 ml/min) to 30 min (20.67±1.86 ml/min, $p<0.0001$) and 70 min (14.23±2.19 ml/min, $p<0.0001$) of reperfusion (Figure 6.3).

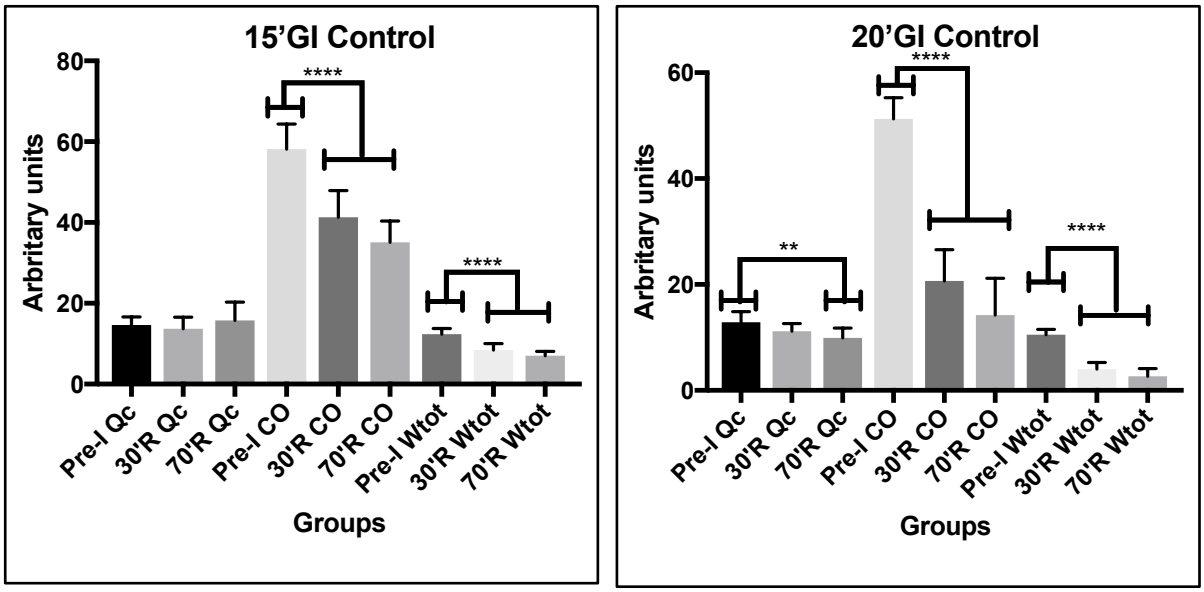
Wtot also decreased significantly, following 15 min of global ischemia, when comparing the pre-I (12.38±0.44 mWatts) interval to 30 min (8.44±0.51 mWatts, $p<0.0001$) and 70 min (6.96±0.33 mWatts, $p<0.0001$) reperfusion. The reduction in total work from pre-I (10.50±0.33) was also significant after 30 min (4.02±0.40 mWatts, $p<0.0001$) and 70 min (2.69±0.45 mWatts, $p<0.0001$), following 20 min of global ischemia (Figures 6.2 and 6.3).

The reduction in mechanical function was statistically more significant after 20 min of global ischemia than when compared to 15 min of ischemia, for both CO and Wtot, at both reperfusion intervals (Figures 6.4-6.5 and Table 6.1). There were however no significant differences between 30 min and 70 min reperfusion in terms of CO ($p=0.055$) or Wtot ($p=0.075$) after both 15 and 20min GI (Figure 6.2 and 6.3).

Table 6.1: Comparison of the mean, SEM and p values for CO and Wtot after 15 and 20 min of global ischemia followed by 30 and 70 min of reperfusion respectively

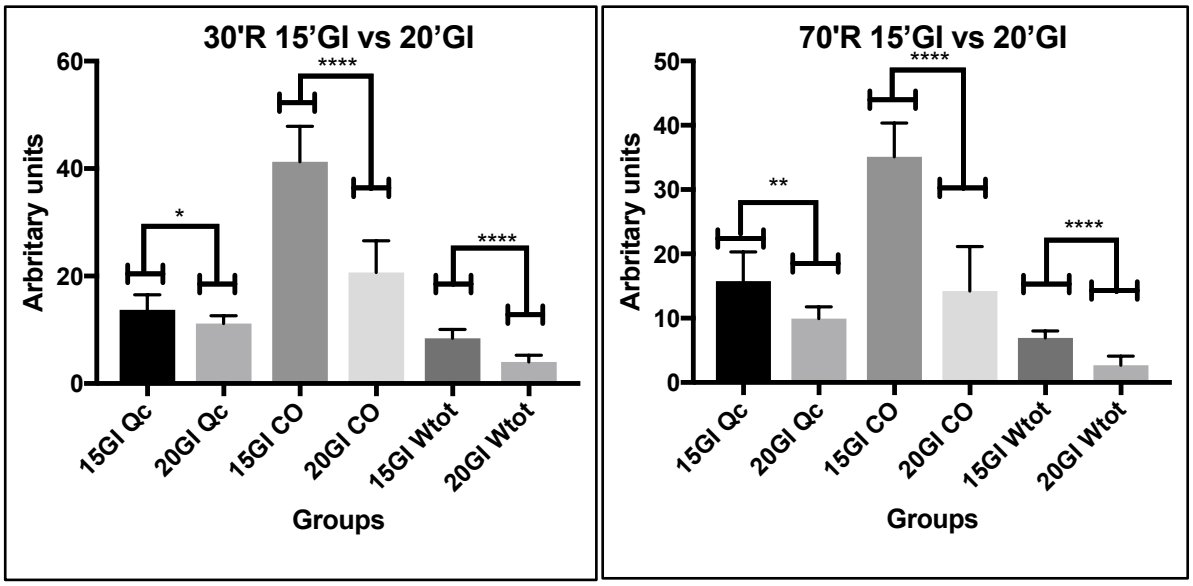
Reperfusion interval	Parameter	20min GI	15min GI	P value
30min	CO	20.67±1.86	41.4±2.08	<0.0001
	Wtot	4.02±0.4	8.44±0.51	<0.0001
70min	CO	14.23±2.19	35.1±1.66	<0.0001
	Wtot	2.69±0.45	6.96±0.33	<0.0001

A similar statistical significant reduction (p<0.0001) in both CO and Wtot was found, following 20 min versus 15 min global ischemia. (n=5-10) Abbreviations: min: minutes, CO: cardiac output, Wtot: total work, SEM: standard error of the mean.



Figures 6.2 and 6.3: Rat heart perfusion data following 15 and 20 minutes global ischemia, reported at time intervals pre-ischemia (15 min into stabilisation), 30 and 70 minutes reperfusion

Data presented as mean ± SEM. ** p< 0.01, **** p<0.0001 (n=5-10) Arbitrary units on the Y axis represent ml/min for Qc and CO and mWatts for Wtot. Abbreviations: Qc: coronary flow rate, Wtot: work total, CO: cardiac output, R: reperfusion, GI: global ischemia and Pre-I: pre-ischemic interval.



Figures 6.4 and 6.5: Rat heart perfusion data comparing cardiac function between 15 and 20 minutes of global ischemia at 30 minutes and 70 minutes reperfusion

Data presented as mean ± SEM. * p < 0.05, ** p< 0.01, **** p<0.0001 (n=5-10) Arbitrary units on the Y axis represent ml/min for Qc and CO and mWatts for Wtot. Abbreviations: Qc: coronary flow rate, Wtot: work total, CO: cardiac output, R: reperfusion, GI: global ischemia and Pre-I: pre-ischemic interval.

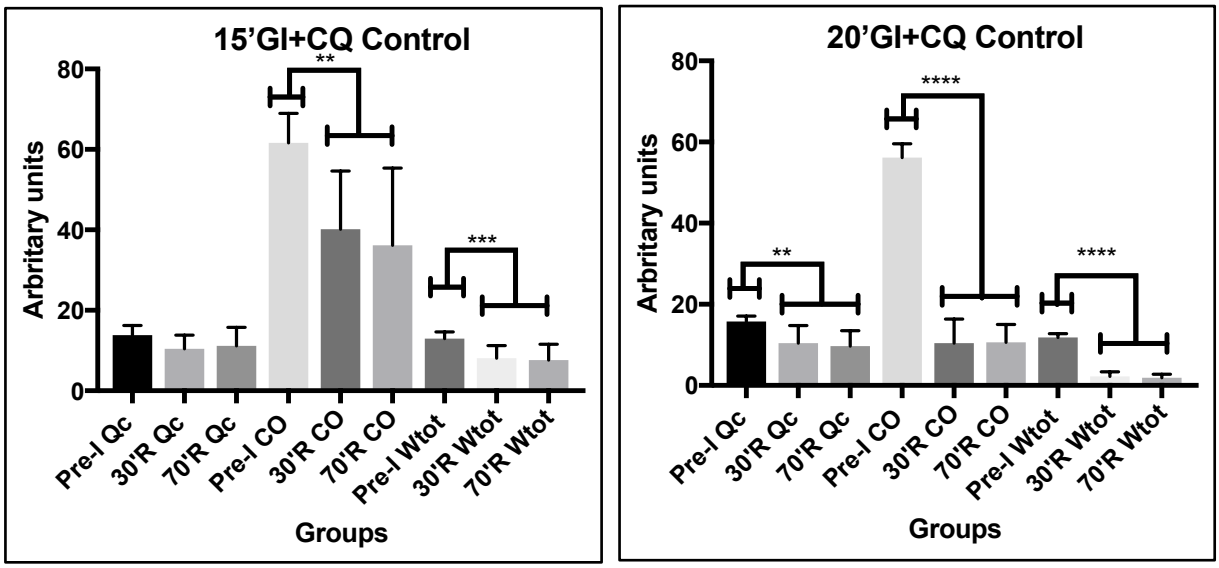
6.2.2. Control experiments: 15 min and 20 min of global ischemia plus Chloroquine

The same significant reduction in CO and Wt during reperfusion after both periods of GI were seen in the groups receiving Chloroquine pretreatment. The drug was therefore not responsible for a deviation from the previously obtained results in the control experiments. In comparing the reperfusion intervals with the pre-ischemic time interval, a significant decrease in CO and Wtot remained for both reperfusion intervals, with the results again being more pronounced in the 20 min global ischemia group, as demonstrated in Table 6.4 below (also refer to Figures 6.6 - 6.9).

Table 6.2: Comparison of the mean, SEM and p values for CO and Wtot after 15 and 20 min of global ischemia plus CQ, followed by 30 and 70 min of reperfusion

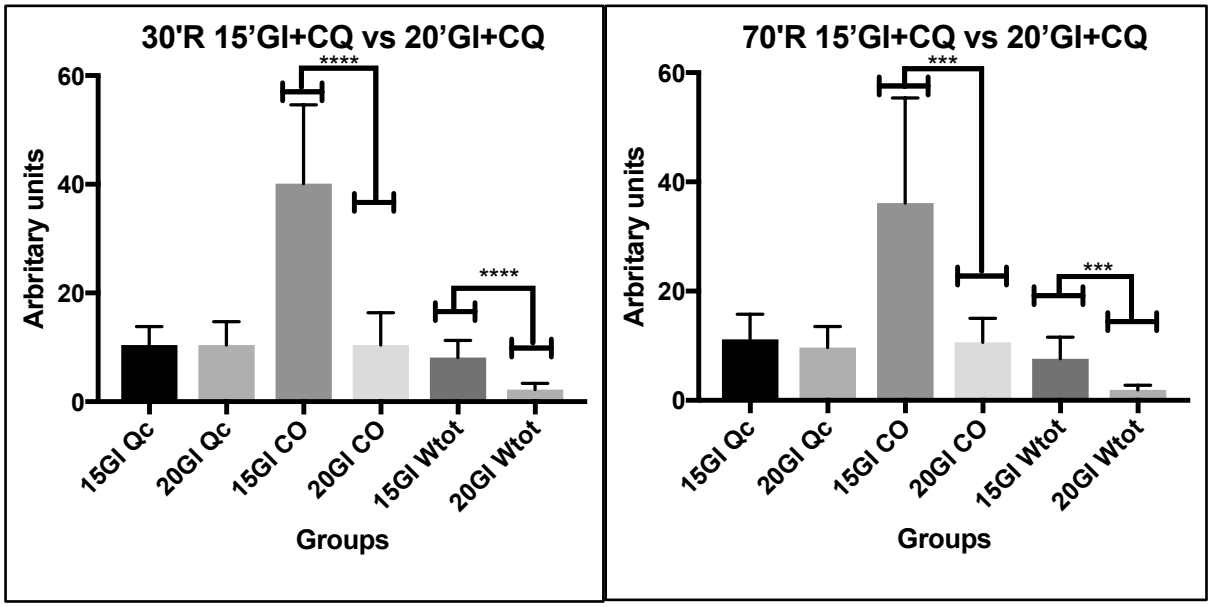
Parameter	GI time +CQ	Pre-I interval	30 min R	P value Pre-I vs 30R	70 min R	P value Pre-I vs 70R
CO	15 min	58.15±1.96	41.40±2.08	=0.0074	35.10±1.66	=0.0016
	20 min	56.15±1.08	10.45±1.88	<0.0001	10.62±1.39	<0.0001
Wtot	15 min	12.95±0.53	8.13±0.99	=0.0047	7.61±1.25	=0.0018
	20 min	11.80±0.31	2.22±0.36	<0.0001	1.91±0.28	<0.0001

Statistical significant decreases (as indicated) observed in both CO and Wtot, following 15 and 20 min of global ischemia, in the 30 min and 70 min reperfusion intervals. (n=5-10) Abbreviations: min: minutes, CO: cardiac output, Wtot: total work, SEM: standard error of the mean.



Figures 6.6 and 6.7: Rat heart perfusion data for 15 and 20 minutes global ischemia, with Chloroquine (CQ), reported at time intervals pre-ischemia (15 min into stabilisation), 30 and 70 minutes reperfusion.

Data presented as mean ± SEM. ** p< 0.01, *** p< 0.001, **** p<0.0001 (n=5-10) Arbitrary units on the Y axis represent ml/min for Qc and CO and mWatts for Wtot. Abbreviations: Qc: coronary flow rate, Wtot: work total, CO: cardiac output, R: reperfusion, GI: global ischemia and Pre-I: pre-ischemic interval.

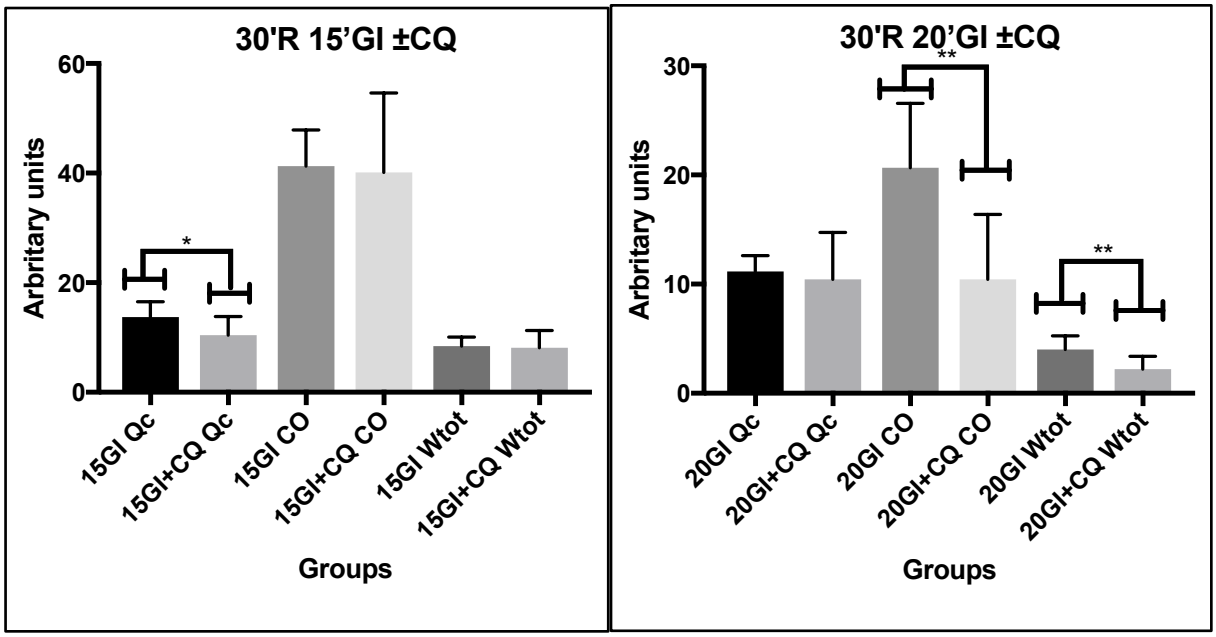


Figures 6.8 and 6.9: Rat heart perfusion data comparing function between 15 and 20 minutes of global ischemia plus Chloroquine (CQ) for 30 minutes and 70 minutes reperfusion

Data presented as mean ± SEM. *** p< 0.001, **** p<0.0001 (n=5-10) Arbitrary units on the Y axis represent ml/min for Qc and CO and mWatts for Wtot. Abbreviations: Qc: coronary flow rate, Wtot: work total, CO: cardiac output, R: reperfusion, GI: global ischemia and Pre-I: pre-ischemic interval.

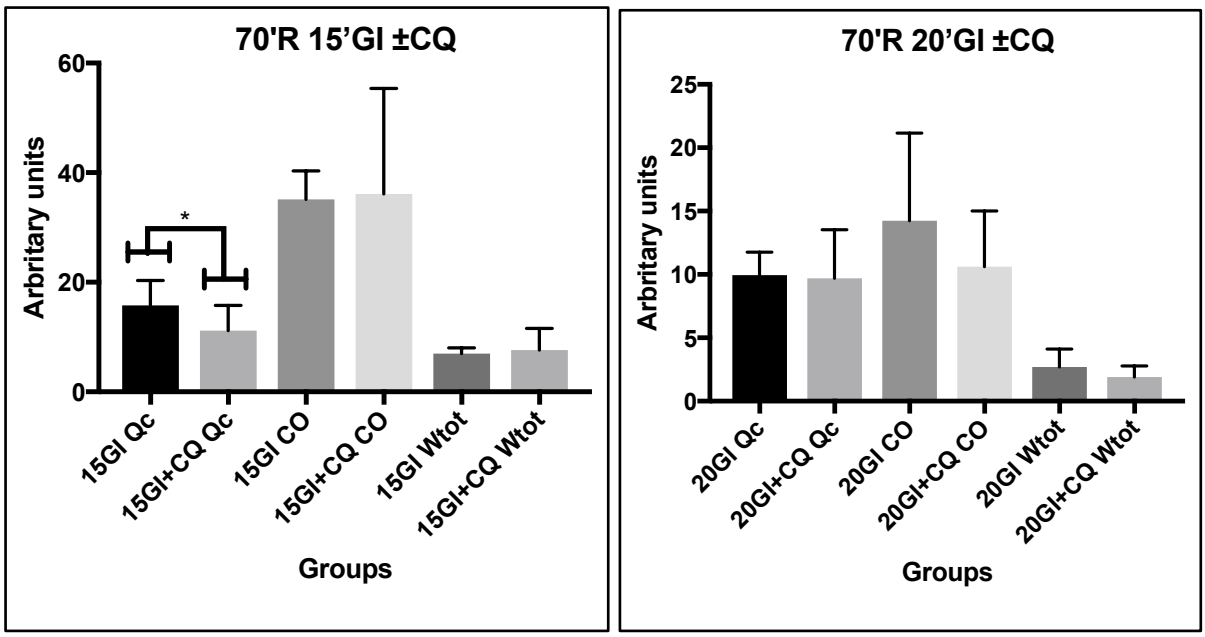
When comparing 15 min of global ischemia with and without CQ, in both reperfusion groups, no statistical significant difference were observed in either CO or Wtot (following 30 min reperfusion: p value for CO±CQ=0.822 and Wtot±CQ=0.783; following 70 min reperfusion: p value for CO±CQ=0.87 and Wtot±CQ=0.62). A decrease in Qc was however found with CQ pretreatment in both the early (13.72±0.001 ml/min vs 10.45±1.07 ml/min, p=0.03) and late (15.75±1.44 ml/min vs 11.15±1.46 ml/min, p=0.038) reperfusion groups (Figure 6.10 and 6.12).

With CQ pretreatment there was a significant decrease in both CO (20.67±1.86 ml/min vs 10.45±1.88 ml/min, p=0.001) and Wtot (4.02±0.39 mWatts vs 2.22±0.37 mWatts, p=0.004) in the 30 min reperfusion following 20 min global ischemia group (Figure 6.11). These changes however disappeared with prolonged reperfusion, at 70 min of reperfusion after 20 min of global ischemia there was no significant change in either CO (p=0.181) or Wtot (p=0.157), compared to its untreated counterparts (Figure 6.13).



Figures 6.10 and 6.11: Rat heart perfusion data comparing function between 15 and 20 min of global ischemia, with and without Chloroquine (CQ) following 30 min of reperfusion

Data presented as mean ± SEM. * p < 0.05, ** p< 0.01 (n=5-10) Arbitrary units on the Y axis represent ml/min for Qc and CO and mWatts for Wtot. Abbreviations: Qc: coronary flow rate, Wtot: work total, CO: cardiac output, R: reperfusion and GI: global ischemia



Figures 6.12 and 6.13: Rat heart perfusion data comparing function after 15 and 20 min of global ischemia, with and without Chloroquine (CQ) following 70 min of reperfusion

Data presented as mean ± SEM. * $p < 0.05$ (n=5-10) Arbitrary units on the Y axis represent ml/min for Qc and CO and mWatts for Wtot. Abbreviations: Qc: coronary flow rate, Wtot: work total, CO: cardiac output, R: reperfusion and GI: global ischemia

6.2.3. Interventional experiments: 3 Methyl-adenine with and without Chloroquine

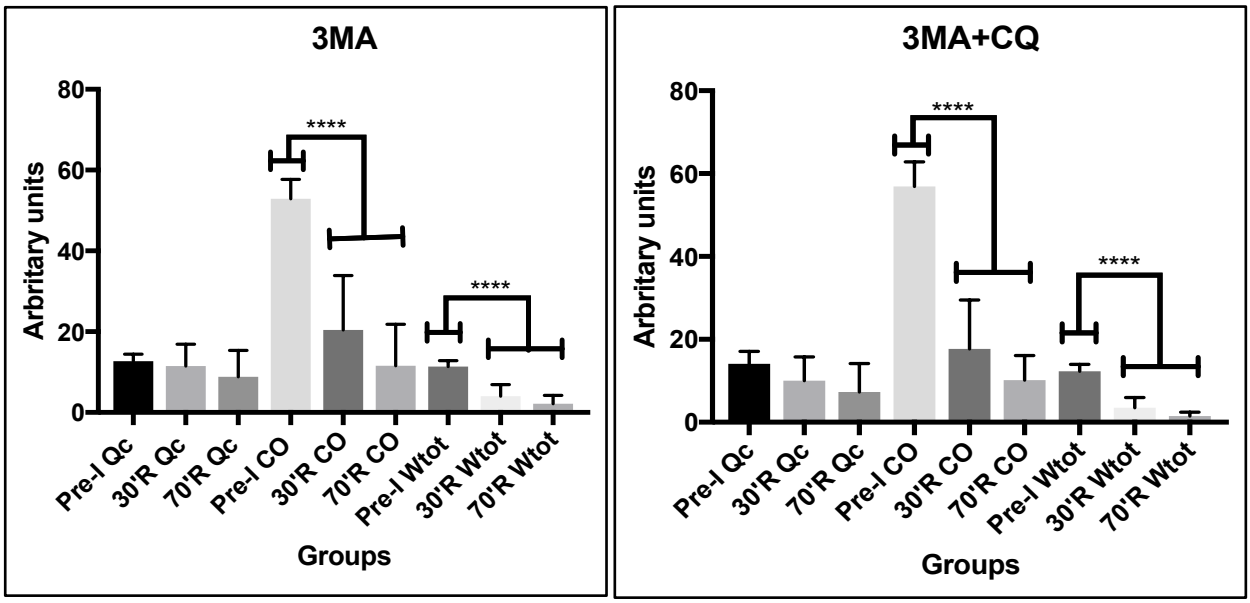
The global ischemic time during the interventional experiments were 20 min. Drugs administration lasted from 0-10 min reperfusion in the early reperfusion group and from 50-60 min in the late reperfusion group. In the extended exposure Rapamycin group administration take place from 0-30 min and from 50-80 min in the early and late reperfusion group respectively.

Treatment with 3 Methyl-adenine, irrespective of the time of administration (both with and without Chloroquine), did not cause a deviation from the previously mentioned control results, with regard to CO and Wtot at 30 min and 70 min of reperfusion (Figures 6.14 and 6.15). The reduction in CO from pre-ischemic interval (52.90±1.52 ml/min) to 30 min (20.42±4.25 ml/min) and 70 min (11.54±4.59 ml/min) reperfusion had the same statistical significance ($p<0.001$) as what was found in the control experiments. Wtot at 30 min (4.03±0.90 mWatts) and 70 min (2.17±0.91 mWatts) reperfusion when compared to the pre-ischemic (11.38±0.47 mWatts) obtained values, had similar statistical significance ($p<0.001$). The addition of pre-experimental CQ also did not result in a deviation from the above results. (CO in ml/min: Pre-ischemic 56.90±1.87 to 30 min 17.63±3.74 and 70 min 10.10±2.67 reperfusion $p<0.001$; Wtot in mWatts: Pre-ischemic 12.27±0.54 to 30 min 3.48±0.83 and 70 min 1.53±0.42 reperfusion $p<0.001$) (Figure 6.15).

6.2.4. Interventional experiments: 1nM Rapamycin with and without Chloroquine

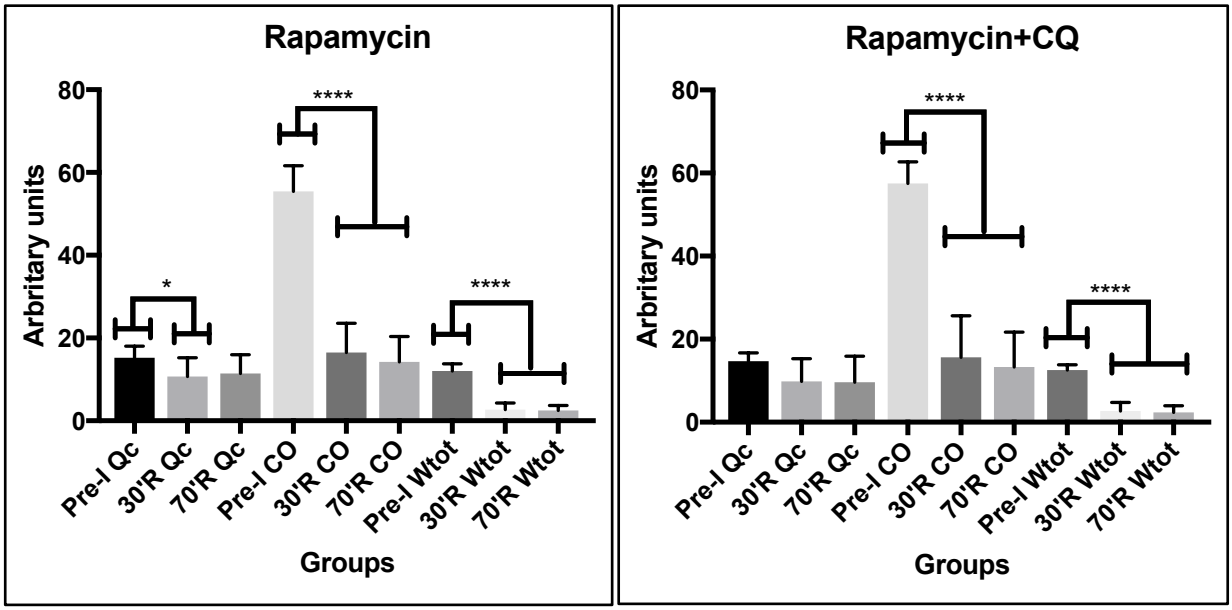
The administration of 1nM Rapamycin during early (CO at 30 min reperfusion 16.42±2.24 ml/min, Wtot at 30 min reperfusion 2.70±0.52 mWatts) and late reperfusion (CO at 70 min reperfusion 14.24±2.73ml/min, Wtot at 70 min reperfusion 2.51±0.54 mWatts) again resulted in the same change ($p<0.001$) when compared to pre-ischemic values for CO (55.45±1.96 ml/min) and Wtot (12.00±0.57 mWatts) (Figure 6.16).

Intraperitoneal Chloroquine was (again) not responsible for a deviation in above results. A statistical significant change of the same magnitude ($p<0.001$) was reflected in both the reperfusion intervals for CO and Wtot. (CO in ml/min: Pre-ischemia 57.50±1.65 vs 30 min 15.60±3.17 and 70 min 13.30±3.76 reperfusion; Wtot in mWatts: Pre-ischemia 12.54±0.41 vs 30 min 2.73±0.68 and 70 min 2.37±0.70 reperfusion) (Figure 6.17).



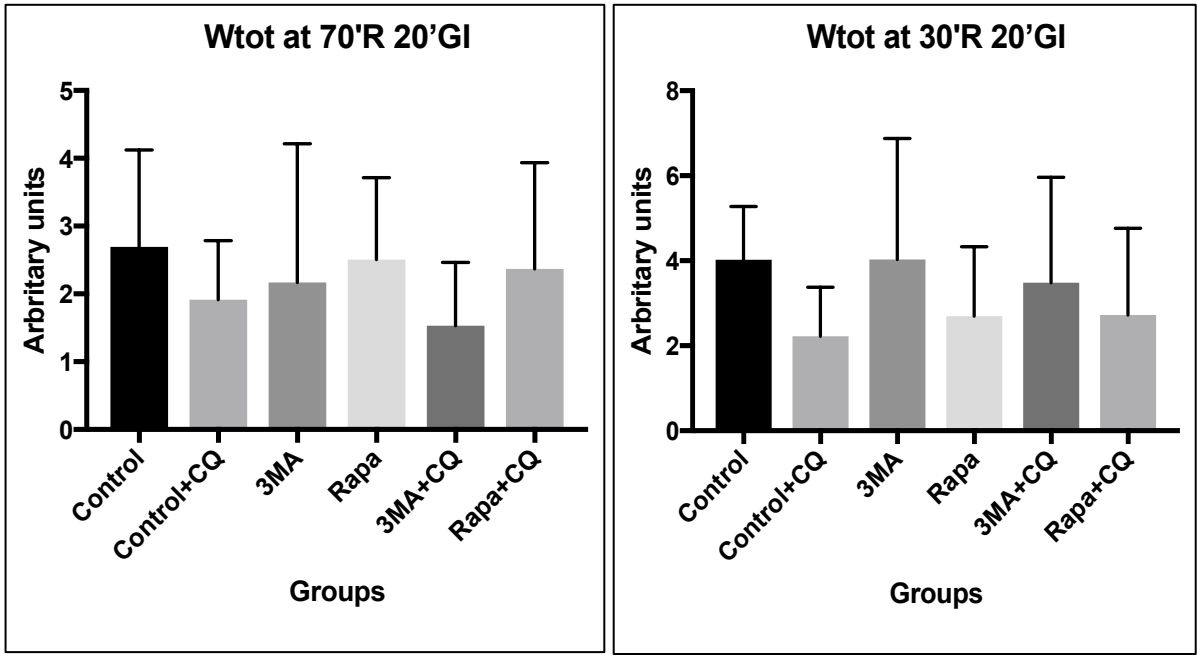
Figures 6.14 and 6.15: Rat heart perfusion data for time intervals pre-ischemia, 30 and 70 minutes reperfusion, with 3 Methyl-adenine (3MA) and 3MA plus Chloroquine (CQ)

Data presented as mean ± SEM. **** p<0.0001 (n=5-10) Arbitrary units on the Y axis represent ml/min for Qc and CO and mWatts for Wtot. Abbreviations: Qc: coronary flow rate, Wtot: work total, CO: cardiac output, R: reperfusion, GI: global ischemia and Pre-I: pre-ischemic interval.



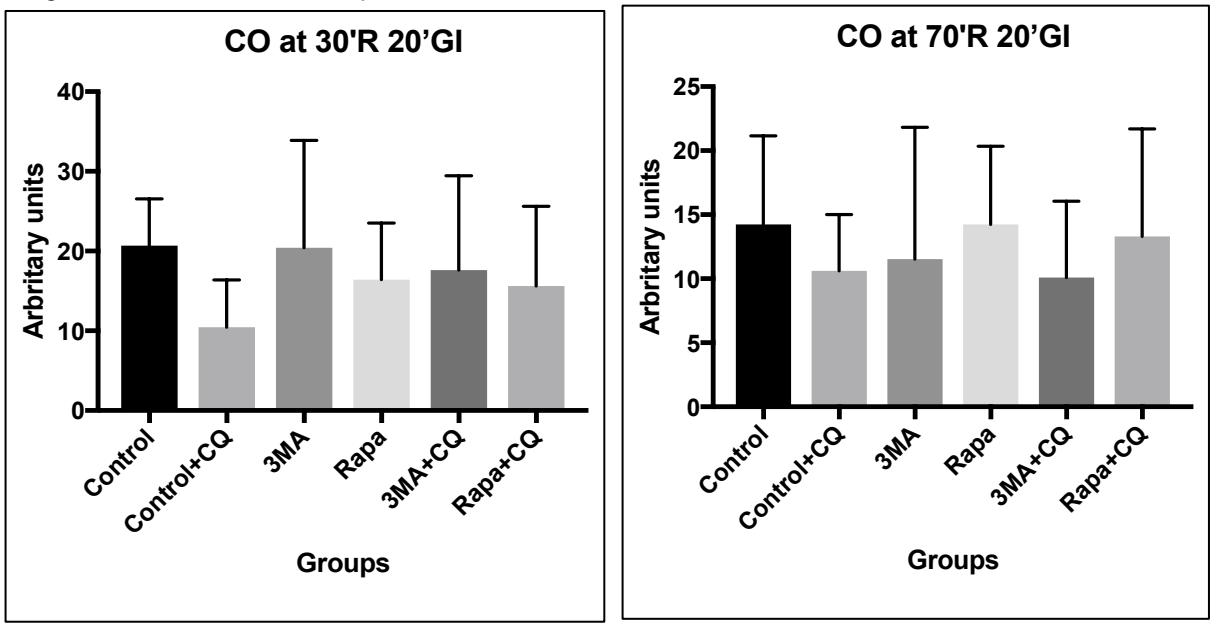
Figures 6.16 and 6.17: Rat heart perfusion data for time intervals pre-ischemia, 30 and 70 minutes reperfusion, with 1 nM Rapamycin and 1 nM Rapamycin plus Chloroquine (CQ)

Data presented as mean ± SEM. * p<0.05 **** p<0.0001 (n=5-10) Arbitrary units on the Y axis represent ml/min for Qc and CO and mWatts for Wtot. Abbreviations: Qc: coronary flow rate, Wtot: work total, CO: cardiac output, R: reperfusion, GI: global ischemia and Pre-I: pre-ischemic interva



Figures 6.18 and 6.19: Rat heart perfusion data; bar graph depicting total work for the different drug groups, at 70 minutes reperfusion (6.18) and 30 min reperfusion (6.19)

Data presented as mean ± SEM. (n=5-10) Arbitrary units on the Y axis represent ml/min for Qc and CO and mWatts for Wtot. Abbreviations: Qc: coronary flow rate, Wtot: work total, CO: cardiac output, R: reperfusion, GI: global ischemia and Pre-I: pre-ischemic interval



Figures 6.20 and 6.21: Rat heart perfusion data, bar graph depicting cardiac output for the different drug groups, at 30 minutes (6.20) and 70 minutes (6.21) reperfusion

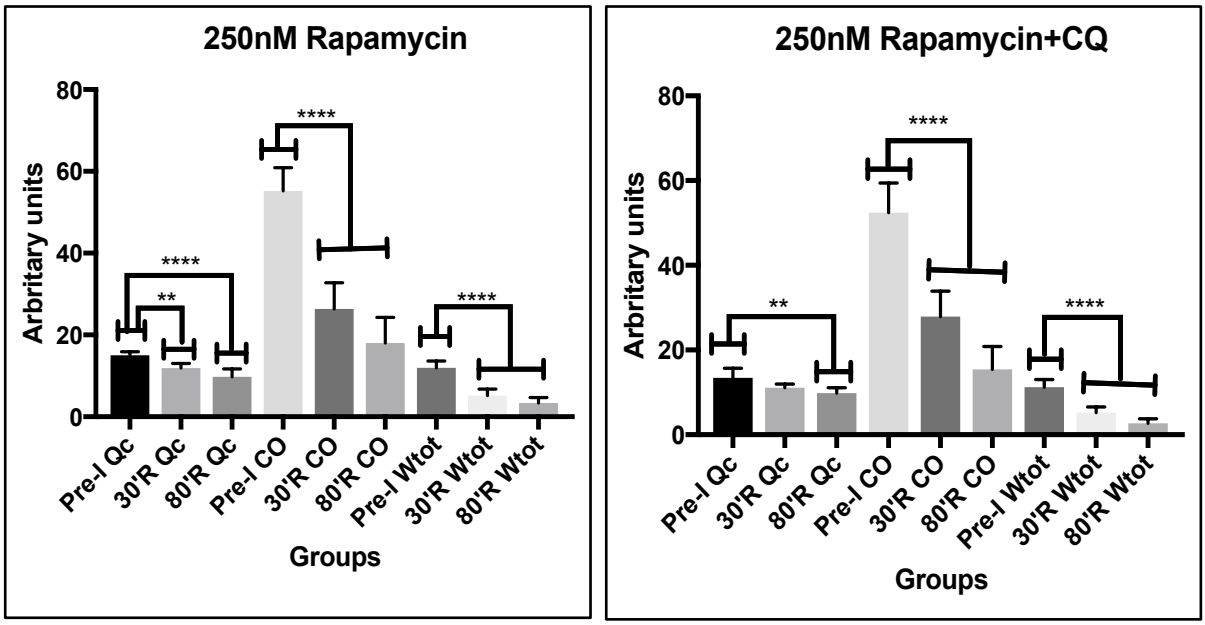
Data presented as mean ± SEM. (n=5-10) Arbitrary units on the Y axis represent ml/min for Qc and CO and mWatts for Wtot. Abbreviations: Qc: coronary flow rate, Wtot: work total, CO: cardiac output, R: reperfusion, GI: global ischemia and Pre-I: pre-ischemic interval.

There was no statistically significant difference in CO and Wtot between any of the drug groups (control, 3MA and 1 nM Rapamycin, all whith and without Chloroquine), at either of the reperfusion periods (Figures 6.18 – 6.21).

6.2.5. Interventional experiments: 250 nM Rapamycin with and without Chloroquine

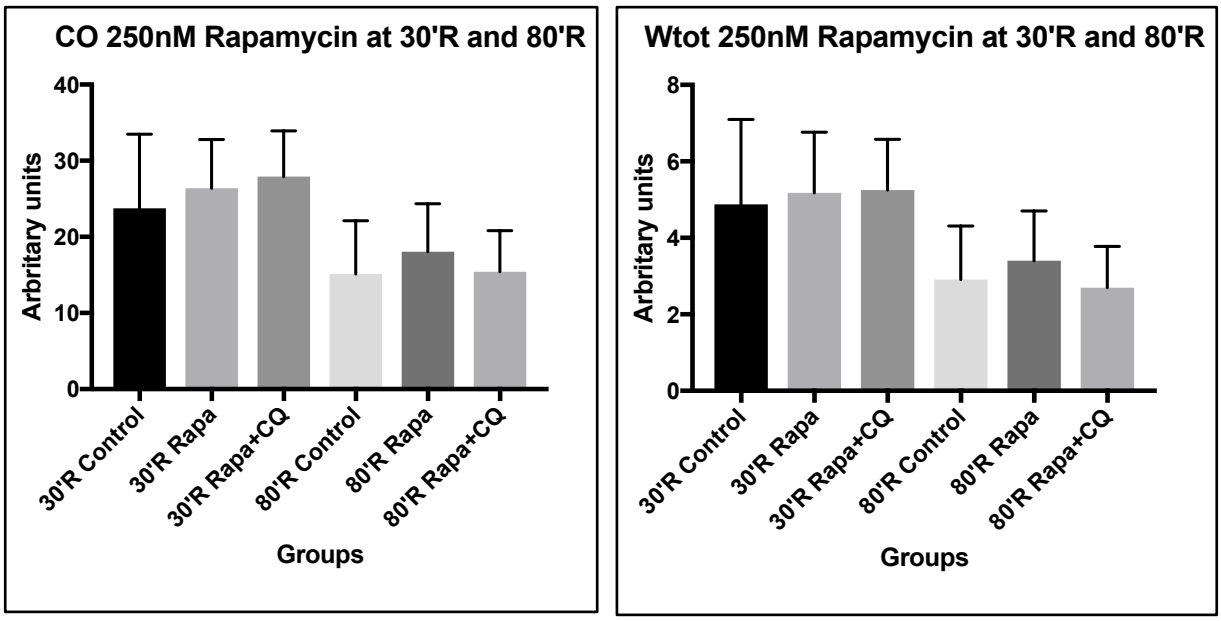
Increasing the Rapamycin dosage and extending the exposure time of Rapamycin to the heart, also did not result in a deviation from the above mentioned results. A significant decrease in CO ($p<0.001$) and Wtot ($p<0.001$) were found when compared to the pre-ischemic levels (CO 55.22±1.89 ml/min, Wtot 11.98±0.55 mWatts), the decrease was similar for early as well as late reperfusion (CO, in ml/min, at 30 min 26.38±3.21 and 80 min 18.00±3.16 reperfusion; Wtot, in mWatts, at 30 min 5.18±0.79 and 80 min 3.40±0.65 reperfusion) (Figure 6.22). In neither of the reperfusion groups did the addition of CQ have an (additional) effect on mechanical function (similar p values). Pre-ischemic CO was 52.40±2.22 ml/min vs 27.90±2.69 ml/min at 30 min and 15.40±2.42 ml/min at 80 min reperfusion. Wtot during the pre-ischemic interval was 11.25±0.57 mWatts, at 30 min and 80 min reperfusion it was 5.25±0.59 mWatts and 2.70±0.48 mWatts respectively (Figure 6.23).

250 nM Rapamycin work heart data was not compared to the rest of the groups since a different perfusion protocol was used to accommodate prolonged drug exposure, as discussed in Chapter 5.



Figures 6.22 and 6.23: Rat heart perfusion data for time intervals pre-ischemia, 30 and 80 minutes reperfusion, with 250 nM Rapamycin and 250 nM Rapamycin plus Chloroquine (CQ)

Data presented as mean ± SEM. (n=5-10) Arbitrary units on the Y axis represent ml/min for Qc and CO and mWatts for Wtot. Abbreviations: Qc: coronary flow rate, Wtot: work total, CO: cardiac output, R: reperfusion, GI: global ischemia and Pre-I: pre-ischemic interval



Figures 6.24 and 6.25: Rat heart perfusion data, bar graph depicting cardiac output (6.24) and total work (6.25) for 250 nM Rapamycin at 30 minutes and 80 minutes reperfusion

Data presented as mean ± SEM. (n=5-10) Arbitrary units on the Y axis represent ml/min for Qc and CO and mWatts for Wtot. Abbreviations: Qc: coronary flow rate, Wtot: work total, CO: cardiac output, R: reperfusion, GI: global ischemia and Pre-I: pre-ischemic interval

In summary, the work heart data illustrate the expected decrease in global myocardial function post global ischemia, this reduction being significantly more in the 20 min vs 15 min global ischemia group. In addition, the time of administration during reperfusion of 3MA and Rapamycin (early or late) did not affect the functional recovery. Likewise, pretreatment of the animals with CQ 1h before experimentation, was without effect on functional recovery, regardless of the presence of interventional drugs.

6.3. WESTERN BLOTTING DATA

The proteins of interest that were analysed during western blotting, following a variety of perfusion protocols and different drug administrations are described in this section. The proteins measured included (also refer to Table 5.2, Chapter 5):

a. Total and phosphorylated ULK1
b. Total and phosphorylated DRP1
c. P62
d. Beclin
e. Rab 9 and
f. LC3 A and B.

Total and phosphorylated ULK1 and DRP1 were analysed and the ratio of the phosphorylated to total protein (ratio = phosphorylated / total) determined to assess activation of the proteins. LC3 ratio equals LC3B / LC3A. The results are presented below, categorized per the protocol followed. For each group, four to five biological samples (n = 4-5 / group) were loaded onto the gels for analysis. A standard control lysate (prepared from untreated rats hearts after being rinsed with KHB, snap frozen and stored at -80°C) was included in each blot for normalisation. The y axis represents arbitrary densitometry units calculated after normalisation of the chemi blots to the stain-free-blots which are also displayed for every set of samples analysed. (* $p < 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p<0.0001$)

In interpretation of the blots, it was kept into consideration that since p62 is incorporated into autophagosomes and released during degradation, its levels are inversely correlated with autophagic activity i.e. *a reduction in its expression indicates increased autophagy* and *vice versa* (Mizushima, Yoshimori and Levine, 2010). Phosphorylated to total (p/t)DRP1 ratio was being interpreted as p62 above (Purnell and Fox, 2013). Increased expression of the other proteins was suggestive of an increase in steady state autophagy (and therefore the number of autophagosomes). In the Chloroquine experiments where blockage of lysosomal degradation occurred, *an increase and accumulation of p62 as well as in the other proteins were interpreted as an increase in activity and therefore increased autophagic flux.* If there was an increase in autophagosomes without the inhibitor (Chloroquine), but not in the presence of the inhibitor, it was interpreted that the autophagosomes accumulated due to impaired fusion and not an increase in flux (Gustafsson and Gottlieb, 2008a; Iwai-Kanai *et al.*, 2008; Gottlieb *et al.*, 2015).

6.3.1. Control experiments

6.3.1.1. 15 min and 20 min global ischemia

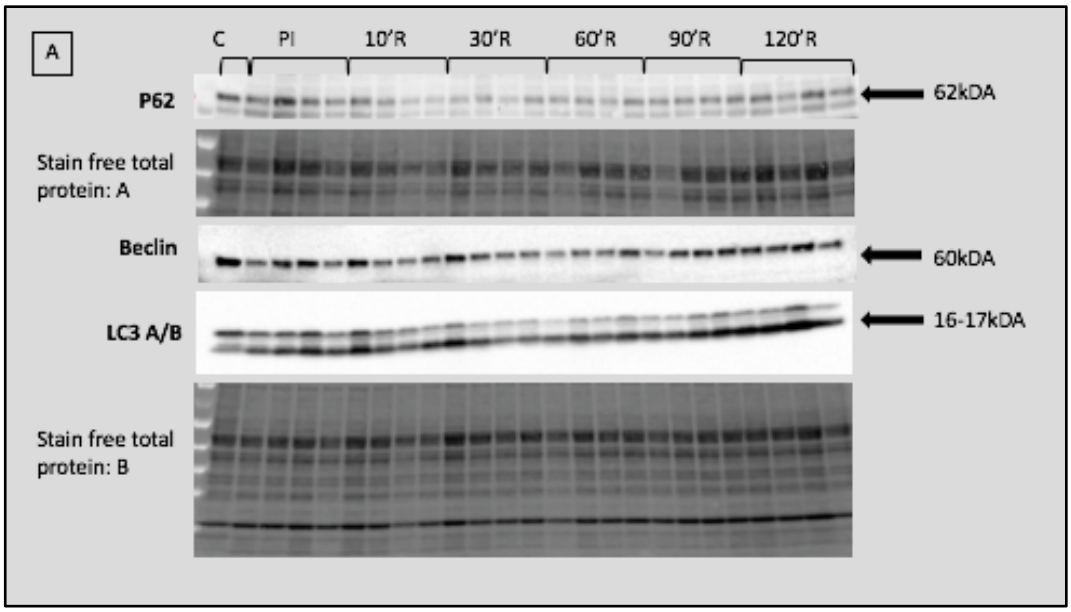


Figure 6.26A: Western blotting data of samples collected during reperfusion after exposure of hearts to 15 min global ischemia, control group. n=4 hearts/group

Western blot chemiluminescent results of P62, Beclin and LC3 A/B, stain-free total protein stain of membranes A and B.

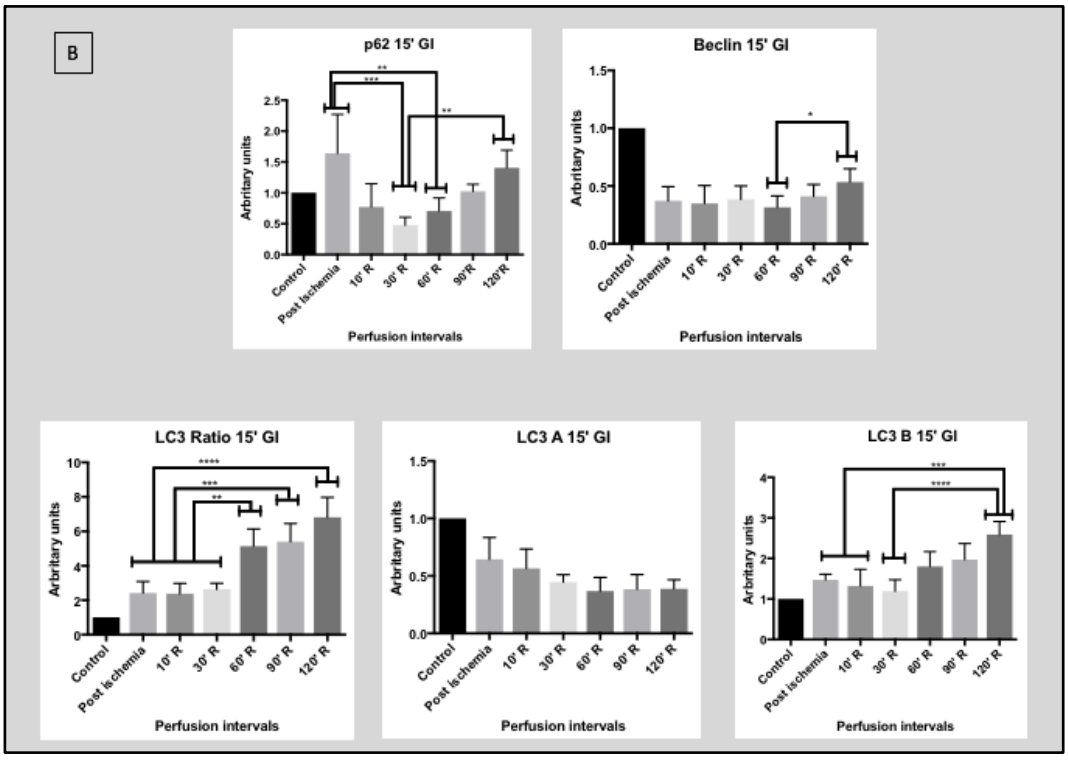


Figure 6.26B: Bar graphs depicting analysed results for p62, Beclin and LC3. Data presented as mean ± SEM (* p < 0.05, ** p< 0.01, *** p< 0.001, **** p<0.0001)

Abbreviations: GI: Global ischemia, C: Control, PI: post-ischemia, R: reperfusion, LC3 ratio = LC3B/LC3A.

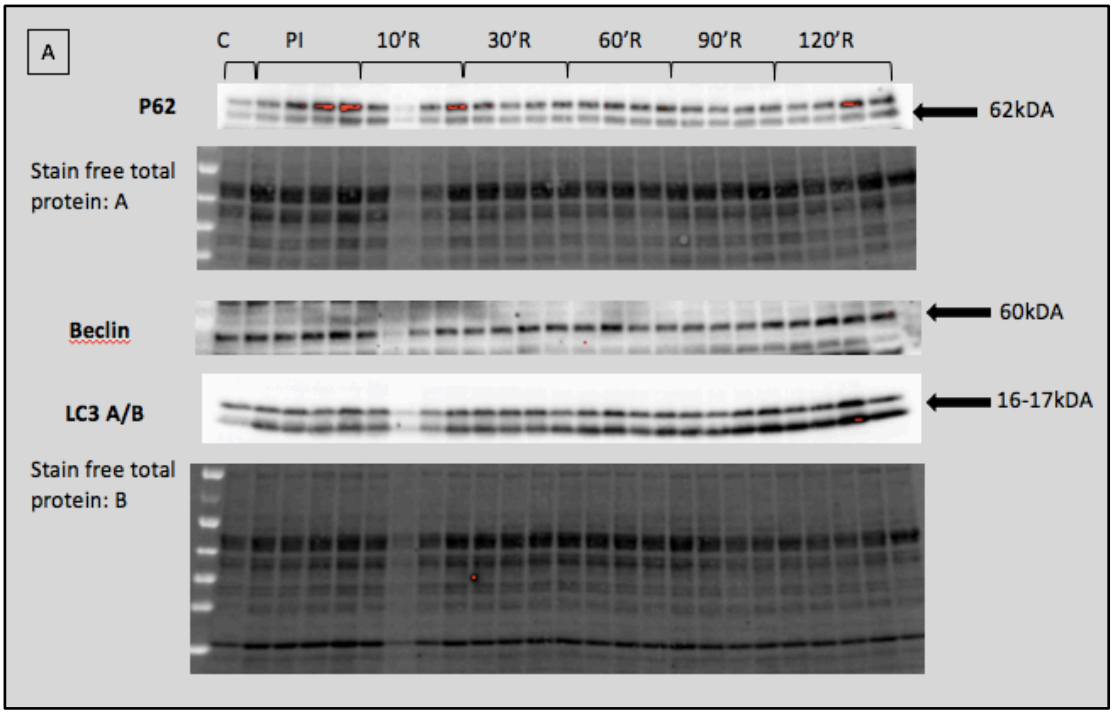


Figure 6.27A: Western blotting data of samples collected during reperfusion after exposure of hearts to 20 min global ischemia, control group. n=4 hearts/group

Western blot results of P62, Beclin and LC3 A/B, stain-free total protein stain of the membranes A and B.

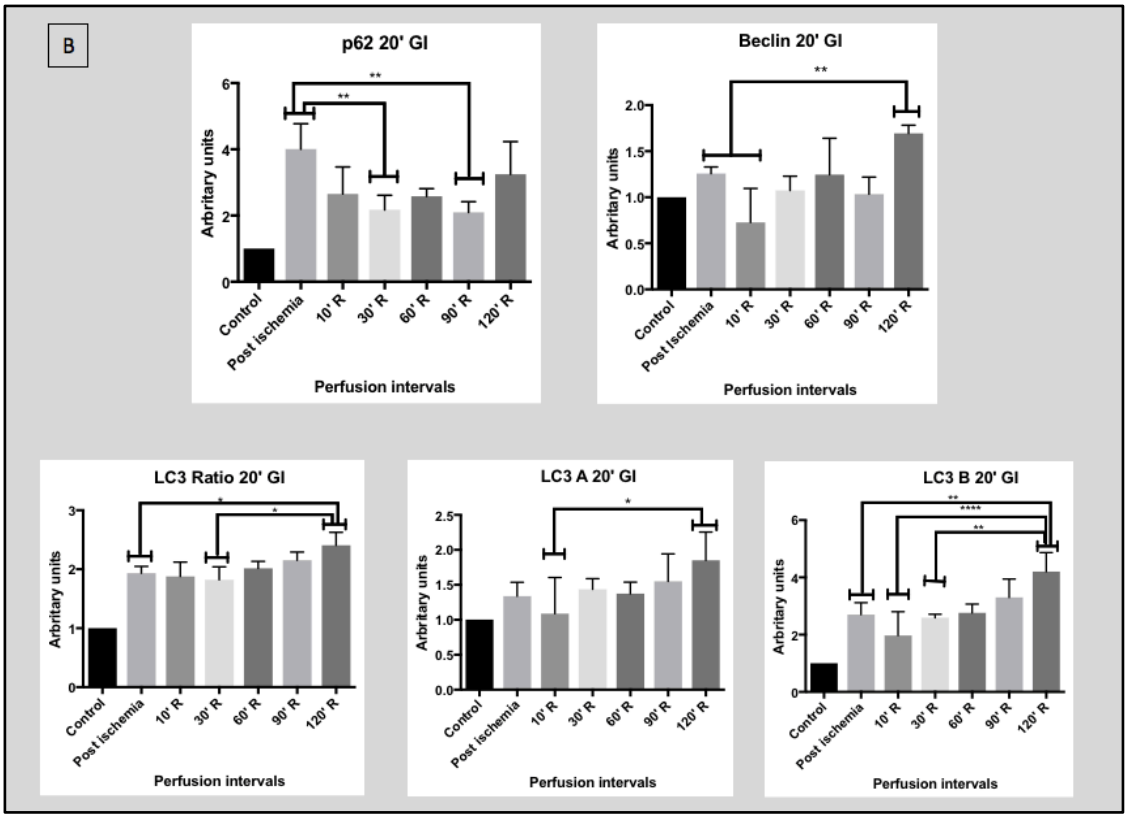


Figure 6.27B: Bar graphs depicting analysed results for p62, Beclin and LC3 A/B. Data presented as mean ± SEM (* p < 0.05, ** p< 0.01, *** p< 0.001, **** p<0.0001)

Abbreviations: Refer to Figure 6.26.

In the above illustrated results for 15 min (Figure 6.26) and 20 (Figure 6.27) min of global ischemia, control experiments, the following were the main findings:

- Using **p62** levels as indicator, a significant increase in autophagic steady state was seen after 15 min ischemia, from the post ischemic (1.64±0,31) interval up to 30 min (0.48±0.06, p=0.01) and 60 min (0.70±0.19, p=0.03) reperfusion. This was followed by a steady decline in autophagy with increased reperfusion time, reaching significance (when compared to 30 min reperfusion) after 120 min reperfusion (30 min reperfusion: 0.48±0.06 vs 120 min reperfusion: 1.41±0.14, p=0.001). There was however no significant difference between 120 min reperfusion and the post ischemic group.
- For **p62** after 20 min of ischemia a significant increase in steady state autophagy was observed at 30 min (2.17±0.22, p=009) and 90 min (2.09±0.16, p=006) reperfusion in comparison to the post ischemic time (4.01±0.44)**.**
- **Beclin** expression suggests a significant increase in autophagy, with increased reperfusion time following 20 min global ischemia (1.26±0.04 vs 1.69±0.05 after post-ischemic interval and 120 min of reperfusion respectively, p=0.008). After 15 min of GI there was a significant increase (p=0.028) in steady state observed between 60 (0.32±0.05) and 120 min (0.53±0.06) of reperfusion. This could be viewed as an *increasing trend* although not a significant increase when compared to the post ischemic time.
- Similar tendencies were observed when using the **LC3 B/A ratio** as indicator of autophagy: An increase in reperfusion time led to a significant increase in LC3 B/A ratio, indicating an increase in autophagy. After 15 min of global ischemia there was a significant increase in LC3 B/A ratio from the post-ischemic time (2.42±0.33) to 60 min (5.14±0.49, p=0.0039), 90 min (5.39±0.53, p=0.0032) and 120 min (6.81±0.58, p=0.006) reperfusion. Following 20 min of global ischemia there was a significant increase in the LC3 B/A ratio from the PI interval to 120 min reperfusion (1.93±0.07 vs 2.41±0.13, p=0.03).
- **LC3 B** illustrated a significant increase in steady state when comparing 120 min reperfusion with the post ischemic group following both 15 (post ischemic 1.47±0.07 vs 120 min reperfusion 2.59±0.16, p=0.0007) and 20 min (post ischemic 2.70±0.21 vs 120 min reperfusion 4.20±0.33, p=0.008) GI.

6.3.1.2. 15 min and 20 min global ischemia with Chloroquine

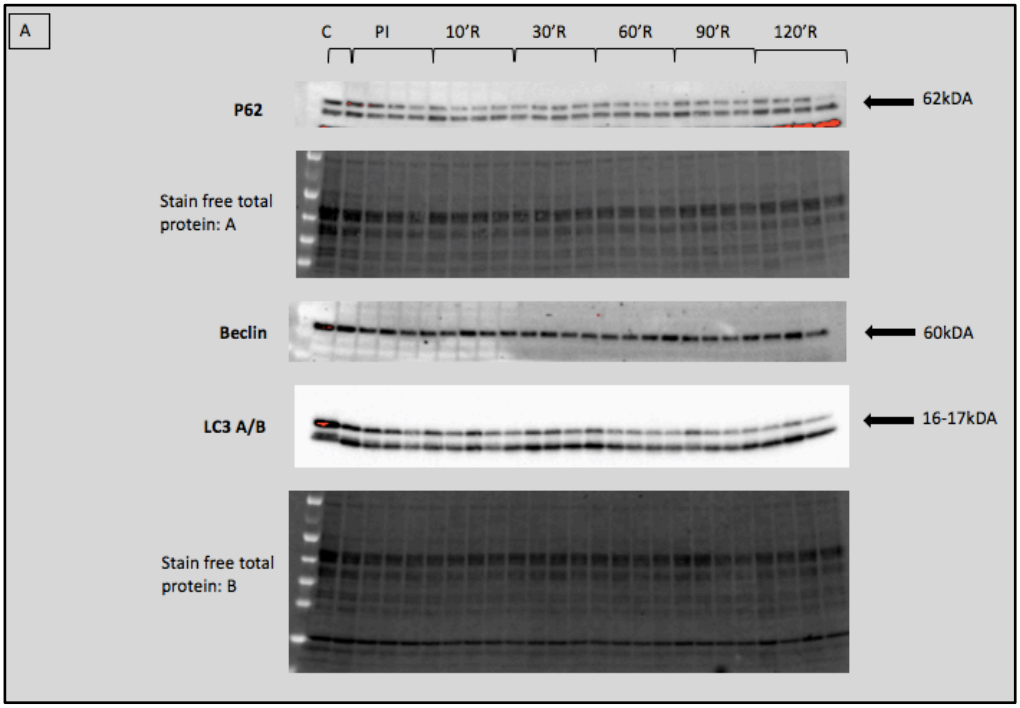


Figure 6.28A: Western blotting data of samples collected during reperfusion after exposure of hearts to 15 min global ischemia, control plus Chloroquine (CQ) group. n=4 hearts/group

Western blot chemiluminescent results of P62, Beclin and LC3 A/B, stain-free total protein stain of membranes A and B.

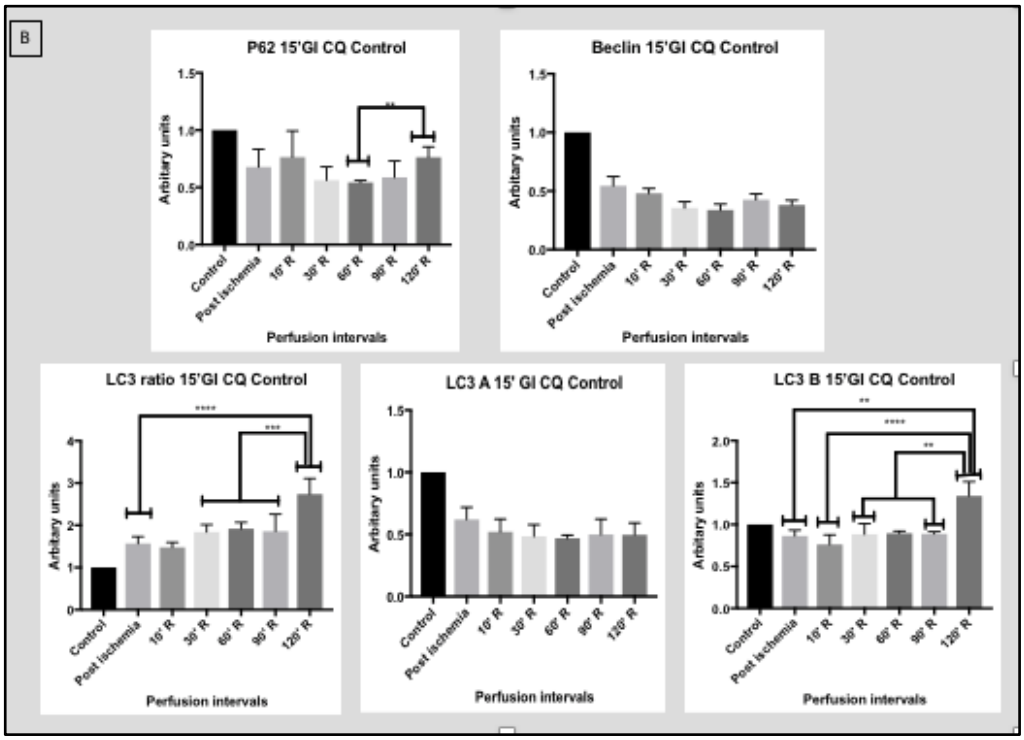


Figure 6.28B: Bar graphs depicting analysed results for p62, Beclin and LC3 A/B. Data presented as mean ± SEM (* p < 0.05, ** p< 0.01, *** p< 0.001, **** p<0.0001)

Abbreviations: Refer to Figure 6.21.

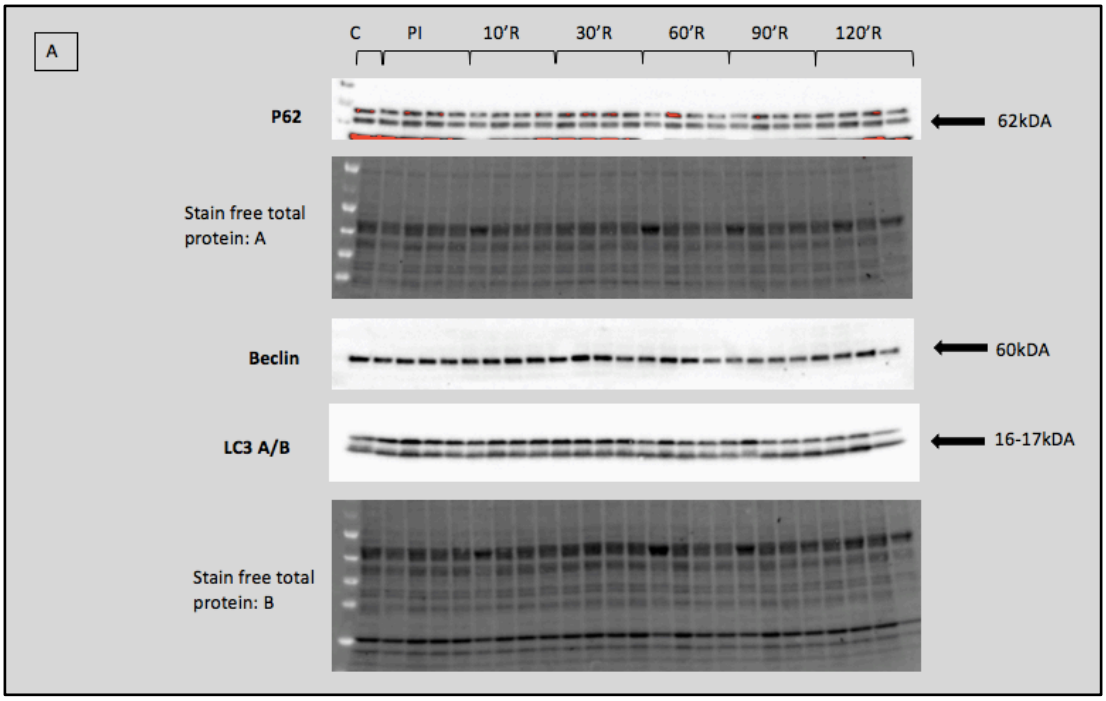


Figure 6.29A: Western blotting data of samples collected during reperfusion after exposure of hearts to 20 min global ischemia, control plus Chloroquine (CQ) group. n=4 hearts/group

Western blot chemiluminescent results of P62, Beclin and LC3 A/B, stain-free total protein stain of membranes A and B.

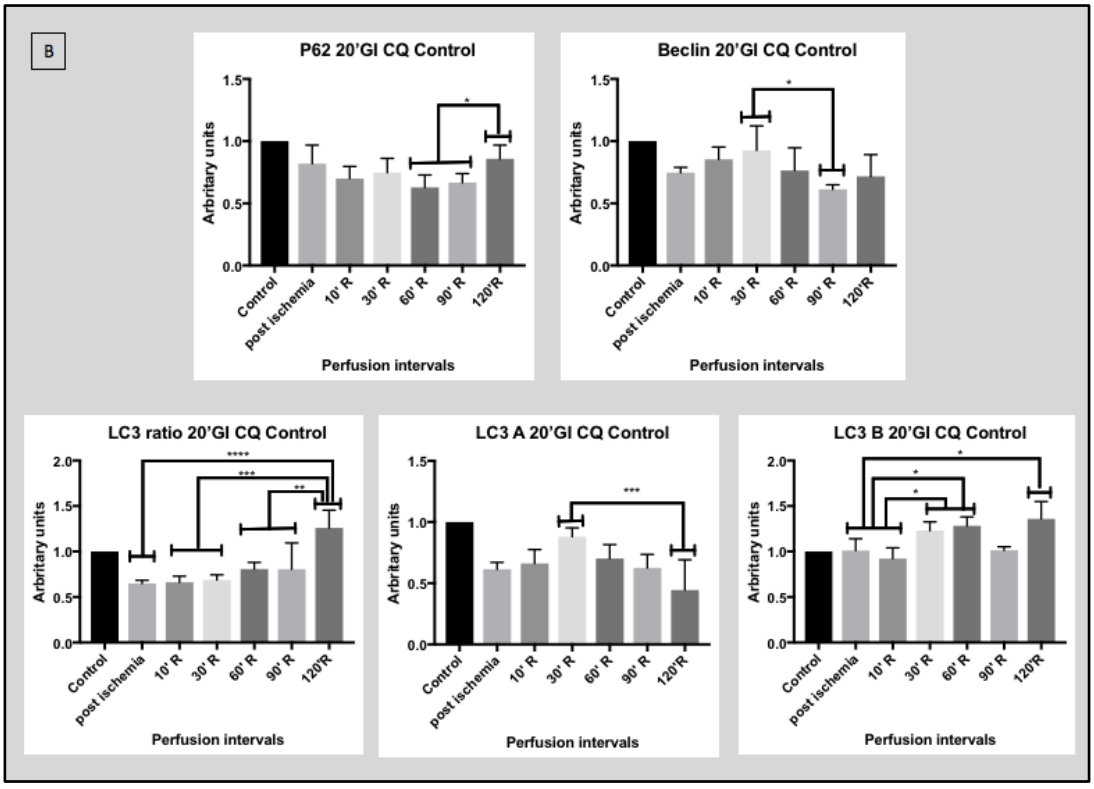


Figure 6.29B: Bar graphs depicting analysed results for p62, Beclin and LC3 A/B Data presented as mean ± SEM (* p < 0.05, ** p< 0.01, *** p< 0.001, **** p<0.0001)

Abbreviations: Refer to Figure 6.21.

Upon summarising the results obtained for 15 (Figure 6.28) and 20 (Figure 6.29) min of global ischemia after pretreatment with Chloroquine, the following noteworthy observations were made:

- With an increase in reperfusion time (from 60 to 120 min), following 15 min (0.63±0.05 vs 0.86±0.06, p=0.02) and 20 min (0.54±0.01 vs 0.76±0.05, p=0.003) global ischemia, significant increases in **p62** occurred, suggesting increased autophagic *flux*.
- Although **Beclin** resulted in a significantly trend increase after 15 min of global ischemia (steady state autophagy), the Chloroquine treatment abolished that change. In the 20 min GI group, there was an initial insignificant increase in Beclin (post ischemia to 30 min reperfusion, p=0.133) followed by a significant decrease during later reperfusion (30 min to 90 min reperfusion, 0,93±0,1 vs 0,61±0,02 respectively, p=0.02) and no change during late (120 min) reperfusion.
- The **LC3 ratio** demonstrated a widespread significant increase in autophagic flux (with an increase in reperfusion time) in both ischemic groups. After 15 min of ischemia this increase was seen between the PI time (1.56±0.08) and 120 min (2.74±0.19, p=0.0008) of reperfusion. The same was true following 20 min of ischemia; with the most significant increase between PI interval and 120 min reperfusion (1.57±0.01 vs 2.74±0.18, p=0.0012).
- **LC3B** results also suggested a significant increase in autophagic flux as demonstrated by the increase between the post ischemic interval and 120 min reperfusion following 15 min (0.86±0.04 and 1.34±0.09 for PI and 120 min reperfusion respectively, p=0.006) and 20 min (1.01±0.07 and 1.36±0.11 for PI and 120 min reperfusion respectively, p=0.034) of GI.

Table 6.3: Summary of the control experiment results for both ischemic periods, with and without Chloroquine (CQ)

GI +/- CQ	Anti-body	Autophagic steady state			Autophagic flux		
		30 min R	120 min R	Significant trend	30 min R	120 min	Significant trend
15 min	**P62**	**Increase**	Unchanged	Decrease	Unchanged	Unchanged	**Increase**
	Beclin	Unchanged	Unchanged	**Increase**	Unchanged	Unchanged	Unchanged
	LC3B/A	Unchanged	**Increase**	**Increase**	Unchanged	**Increase**	**Increase**
	LC3B	Unchanged	**Increase**	**Increase**	Unchanged	**Increase**	**Increase**
20 min	**P62**	**Increase**	Unchanged	**Increase**	Unchanged	Unchanged	**Increase**
	Beclin	Unchanged	**Increase**	**Increase**	Unchanged	Unchanged	Unchanged
	LC3B/A	Unchanged	**Increase**	**Increase**	Unchanged	**Increase**	**Increase**
	LC3B	Unchanged	**Increase**	**Increase**	**Increase**	**Increase**	**Increase**

The increase or decrease in the autophagic steady state and flux after 30 and 120 min of reperfusion reflects whether there was a statistically significant change between the post-ischemic and the indicated reperfusion period, the trend refers to what happened with an increase in the reperfusion interval. Abbreviations: R: reperfusion, GI: global ischemia, CQ: Chloroquine, LC3 B/A = LC3B/LC3A ratio and min: minutes.

Considering the control results collectively (Table 6.3), a global ischemic time of 20 min was responsible for a more consistent increase in autophagy in comparison with 15 minutes of ischemia. This is demonstrated by the fact that all four the antibodies reflected an increase in steady state autophagy, as well a significant increase in trend following 20 min GI. Three of the four antibodies following 15 min of GI demonstrated an increase in steady state and a significant increase in trend. This clear difference however disappeared when assessing autophagic flux, the same number of antibodies indicated an increase in autophagic flux as well as a significant increase in trend following both 15 min and 20 min global ischemia. A longer reperfusion period (120 min versus 30 min) clearly resulted in a more pronounced increase in autophagy steady state and flux. By far the majority of autophagic changes was found after 120 min of reperfusion. These results were used as the main motivation to employ 20 min for global ischemia, 30 min (instead of 10 min) for early reperfusion and 120 min (instead of 60 or 90 min) for late reperfusion in the subsequent interventional experiments.

6.3.2. Interventional experiments

For the interventional experiments, a number of indicators of alternative autophagic pathways were analysed in addition to the conventional indicators such as p62, LC3 and Beclin. Early reperfusion implies drug administration from 0-10 min of reperfusion and freeze-clamping at 30 min reperfusion. During late reperfusion drugs were administered

from 50-60 min and the heart were freeze-clamped at 120 min reperfusion.

6.3.2.1. Early reperfusion with 3 Methyl-adenine, with and without Chloroquine

Administration of 3MA during the first 10 min of reperfusion, had the following effects, when compared to their untreated counterparts (Figure 6.30 A and B):

- No change in steady state autophagy as assessed by proteins **p62, Beclin, p/tDRP1-, p/tULK1-** and the **LC3 B/A ratio**.
- **Rab9** supports a significant decrease in steady state autophagy (no drug to 3MA 0.93±0.02 vs 0.69±0.06 respectively, p=0.004) as well as autophagic flux (no drug to 3MA plus CQ 0.93±0.02 vs 0.74±0.06 respectively, p=0.016).
- This decrease in flux is supported by the **p62** results, where Chloroquine treatment caused a significant reduction of p62 in the untreated group compared to the 3MA plus CQ (1.54±0.13 vs 0.99±0.05, p=0.0045).
- Another important observation is that 3MA abolished the increase in autophagic steady state and flux observed in the control experiments as supported by p62, Beclin, LC3B and LC3 B/A ratio.

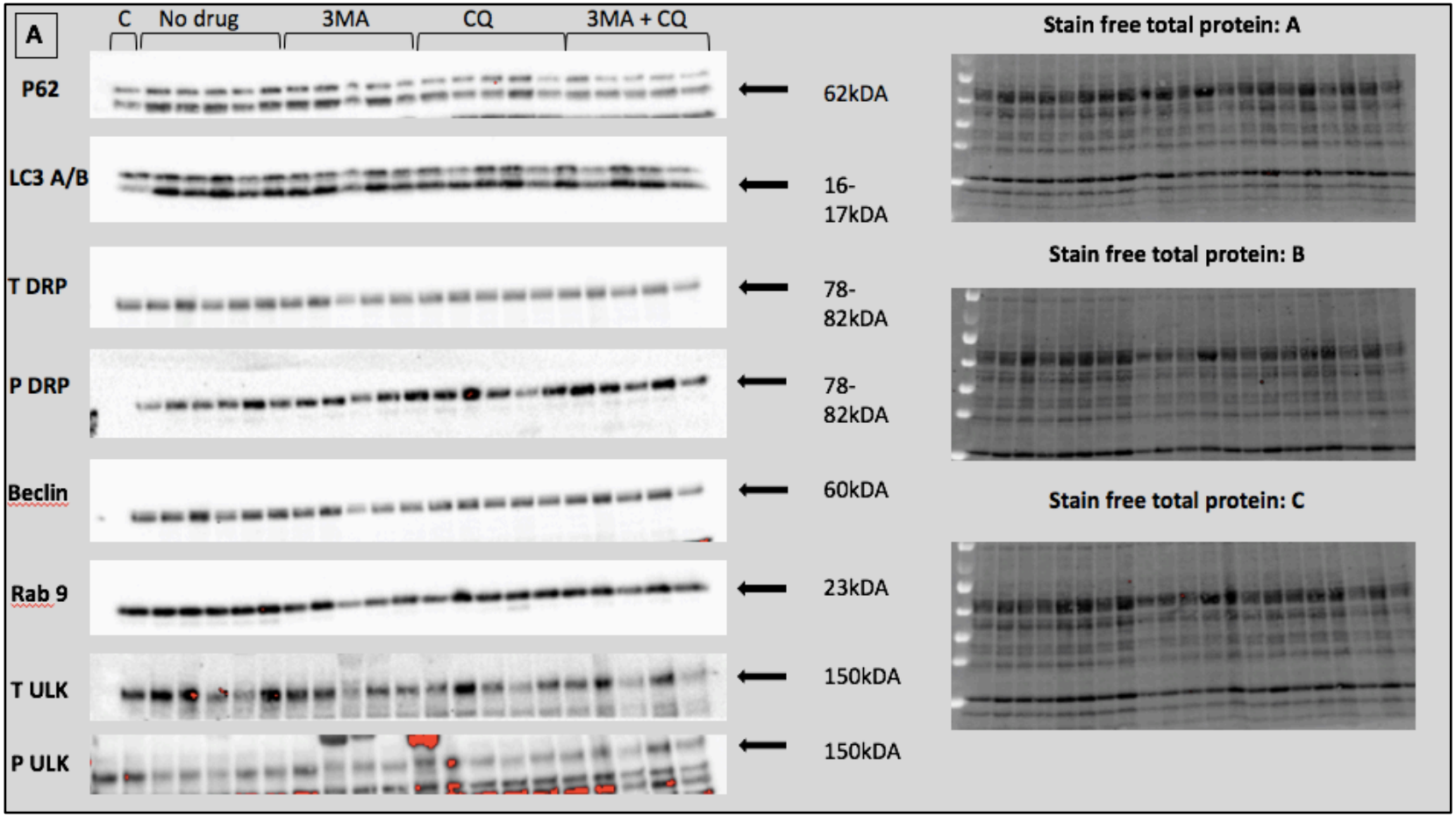


Figure 6.30A: Western blotting data of samples collected during reperfusion after exposure of hearts to 20 min global ischemia and 3MA during early reperfusion ± Chloroquine (CQ) pretreatment. n=5 hearts/group

Protein chemiluminescent results include those of P62, Beclin and LC3 A/B, Rab 9, total (T), phosphorylated (P) ULK1 and DRP1. Stain-free total protein stain of the membranes A, B and C are included. P62 and LC3 from membrane A; T DRP, Beclin, T ULK from membrane B and P DRP and P ULK from membrane C. Abbreviations: 3MA: 3 Methyl-adenine, CQ: Chloroquine, kDA: kilodalton, C: Control, DRP1 ratio = phosphorylated DRP1 /total DRP1, ULK1 ratio = phosphorylated ULK1 /total ULK1, LC3 ratio = LC3B/LC3A.

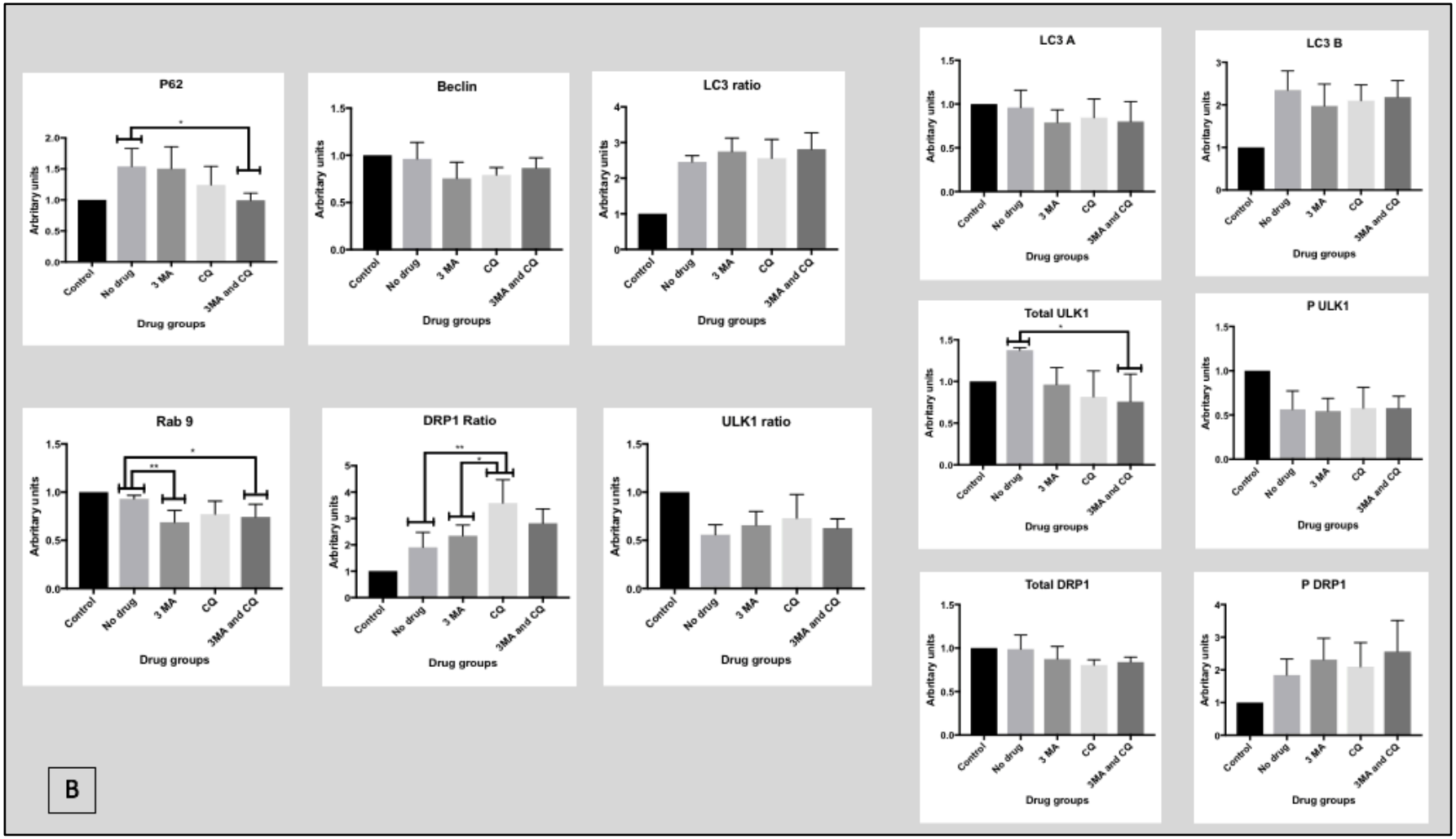


Figure 6.30B: Western blotting data for 3MA, with and without Chloroquine (CQ), early reperfusion group

Bar graphs depicting analysed results for p62, Beclin, Rab 9, LC3 A/B, total (T) and phosphorylated (P) ULK1 and DRP1, and the ratio's for LC3, ULK1 and DRP1. Data presented as mean ± SEM (* $p < 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p<0.0001$)

6.3.2.2. Early reperfusion with 1 nM Rapamycin, with and without Chloroquine.

In the **early reperfusion, Rapamycin, intervention group** (Figure 6.31A and B),

- There was a significant increase from no drug to the combination of Rapamycin and CQ in both **p/tDRP1 ratio** (1.53±0.14 vs 1.91±0.08, p=0.042) and **Beclin** (1.14±0.09 vs 1.68±0.19, p=0.025)**,** representing an increase in flux.
- **p62** was the only protein in this group in which a significant increase in steady state autophagy was implicated by the decreasing levels from no drug to Rapamycin (1.51±0.06 vs 1.08±0.07, p=0.007). However, the CQ data supported a significant decrease in flux (no drug to Rapamycin plus CQ, 1.51±0.06 vs 1.17±0.06, respectively, p=0.014).
- No significant change in steady state or flux were found evaluating the results for Rab9, LC3 B/A- and p/tULK1-ratios.

6.3.2.3. Late reperfusion with 3 Methyl-adenine, with and without Chloroquine

Western blotting results for the **late reperfusion, 3MA, interventional group** (Figure 6.32 A and B) can be summarised as follows:

- The decrease in protein levels for **p/tDRP1 ratio** (1.47±0.05 vs 0.09±0.15, p=0.0136) between no drug and the 3MA group, supported a significant increase in steady state autophagy.
- This was supported by **p/tULK1 ratio** and **LC3B** in which a significant increase in steady state was found between the no drug and 3MA groups (p/tULK1 ratio: 0.40±0.08 no drug to 0.84±0.12 3MA group, p=0.0152 and LC3B: 1.02±0.05 no drug to 1.14±0.03 3MA group, p=0.0001).
- The increase **in P62** (0.90±0.08 vs 1.23±0.12, p=0.055) from no drug to 3MA group however implicates a decrease in steady state of the conventional pathway.
- A significant difference, between no drug and the 3MA and CQ combination, was found in **p/tDRP1 ratio** (1.47±0.05 vs 2.09±0.12, p=0.0041) and **p62** (0.90±0.08 vs 0.69±0.04, p=0.042), which respectively implied an increase and decrease in autophagic flux.
- Neither the **LC3 B/A ratio, Beclin** or **Rab9** demonstrated any significant changes.

3MA administered during late reperfusion resulted in inconclusive results. Although the majority of proteins supported an increase in steady state, no conclusions can be made regarding what happened during flux. From a flux perspective, it did abolish the increase in flux supported by LC3B and LC3 B/A ratio in the control results (Table 6.4).

6.3.2.4. Late reperfusion with 1nM Rapamycin, with and without Chloroquine.

Administration of Rapamycin at a concentration of 1nM administered during late reperfusion (50-60 min) (Figure 6.33 A and B), was less effective in increasing autophagy than the late reperfusion untreated control group. The most important observations were:

- The increase between the no drug and the Rapamycin groups was significant for both **Rab9** (1.49±0.19 vs 2.19±0.16 respectively, p=0.0293) and the **p/tDRP1 ratio** (0.45±0.04 vs 0.74±0.05, p=0.004).
- The **p/tDRP1 ratio,** with a significant increase between no drug and the Rapamycin plus CQ group (0.45±0.04 vs 0.78±0.07, p=0.006) was the only protein supporting an increase in autophagic flux.
- The addition of Rapamycin did not result in any significant change in **P62**, **LC3 B/A ratio** and **Beclin levels**.

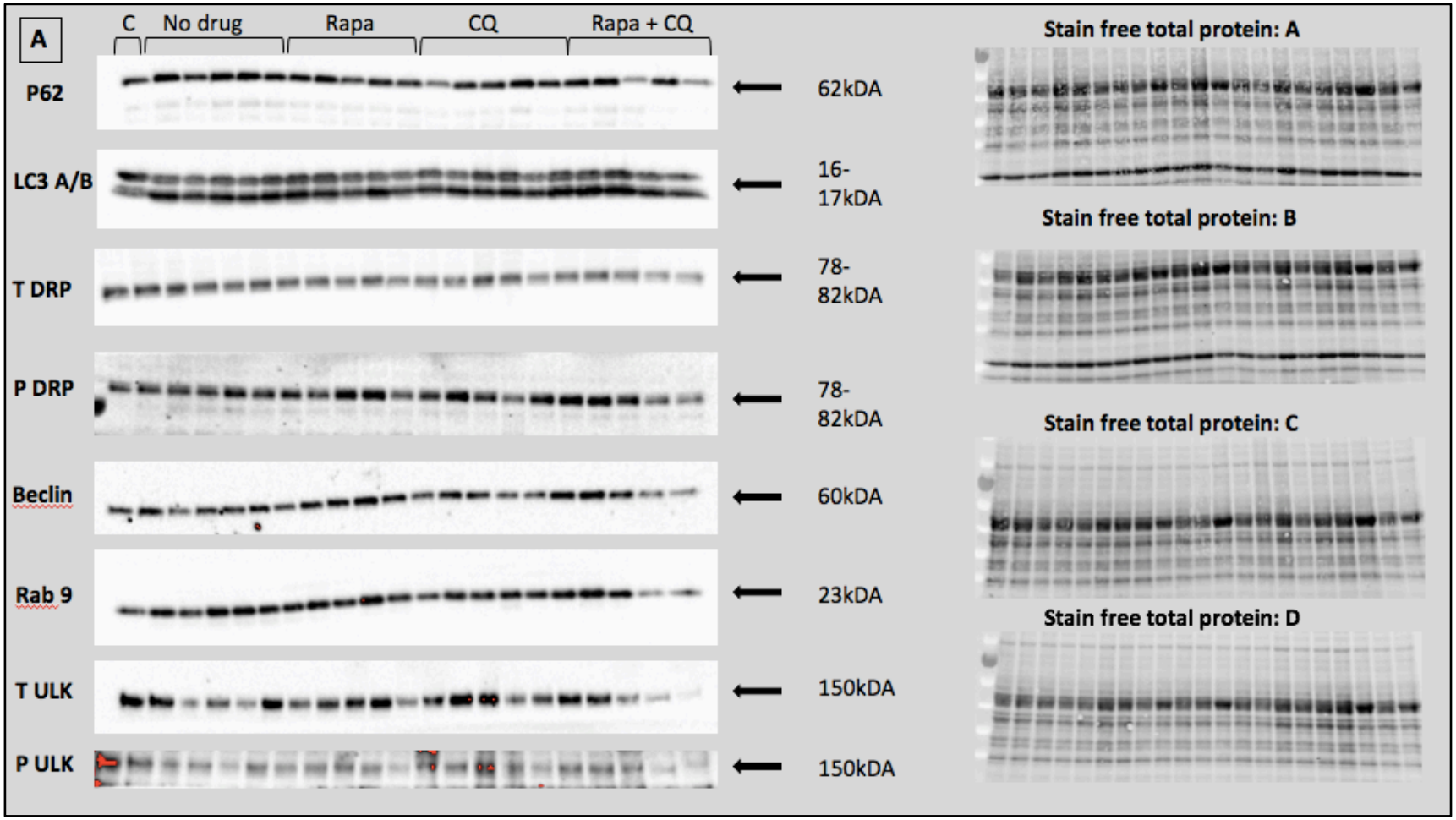


Figure 6.31A: Western blotting data of samples collected during reperfusion after exposure of hearts to 20 min global ischemia and 1nM Rapamycin (rapa) during early reperfusion ± Chloroquine (CQ) pretreatment. n=5 hearts/group

Protein chemiluminescent results include those of P62, Beclin and LC3 A/B, Rab 9, total (T) and phosphorylated (P) ULK1 and DRP 1. Stain-free total protein stain of the membranes A, B, C and D are included. P62 from membrane A; Beclin and Rab 9 from membrane B; LC3, T ULK and T DRP from membrane C and P DRP and P ULK from membrane D. Abbreviations: refer to Figure 6.30 A.

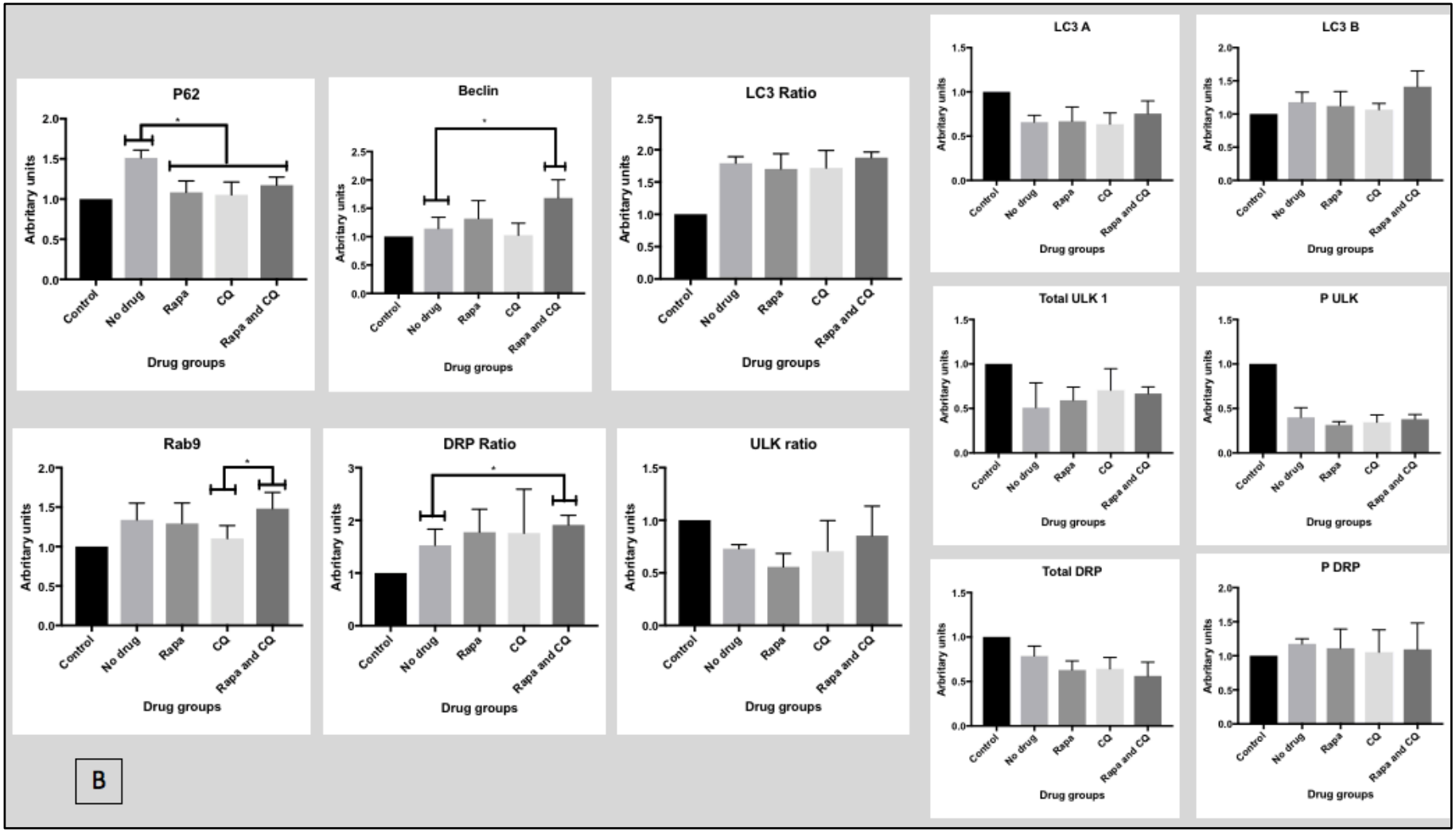


Figure 6.31B: Western blotting data for the 1 nM Rapamycin (Rapa) with and without Chloroquine (CQ), early reperfusion group
Bar graphs depicting analysed results for p62, Beclin, Rab 9, LC3 A/B, total (T) and phosphorylated (P) ULK1 and DRP1, and the ratio's for LC3, ULK1 and DRP1. Data presented as mean ± SEM (* $p < 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p<0.0001$)

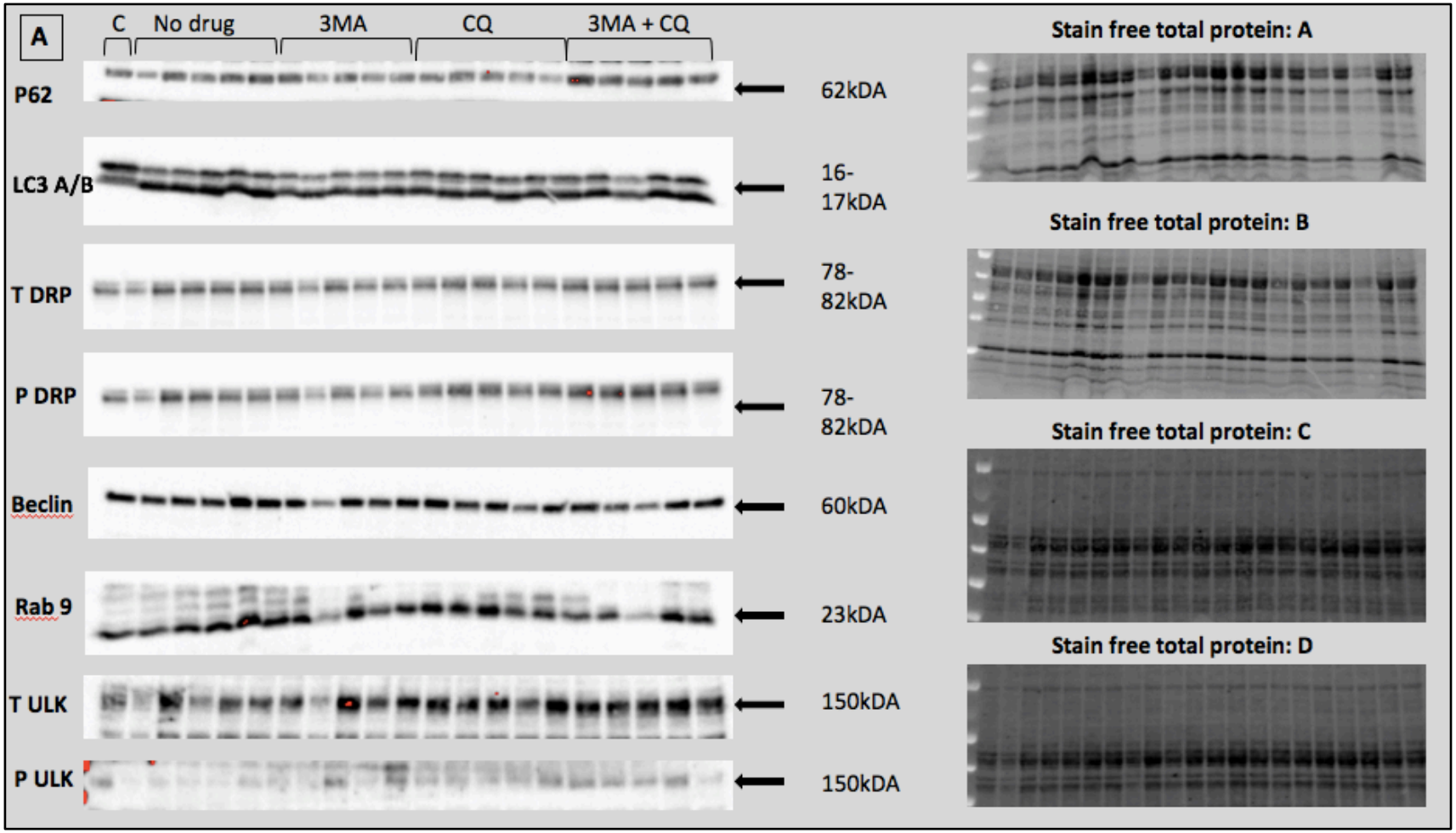


Figure 6.32A: Western blotting data of samples collected during reperfusion after exposure of hearts to 20 min global ischemia and 3MA during late reperfusion ± Chloroquine (CQ) pretreatment. n=5 hearts/group

Western blot chemiluminescent results of P62, Beclin and LC3 A/B, Rab 9, Total (T), phosphorylated (P) ULK1 and DRP 1. Stain-free total protein stain of the membranes A, B, C and D. LC3 from membrane A, Beclin and Rab 9 from membrane B, T ULK, T DRP and p62 from membrane C and P DRP and P ULK from membrane D. Abbreviations: refer to Figure 6.30 A.

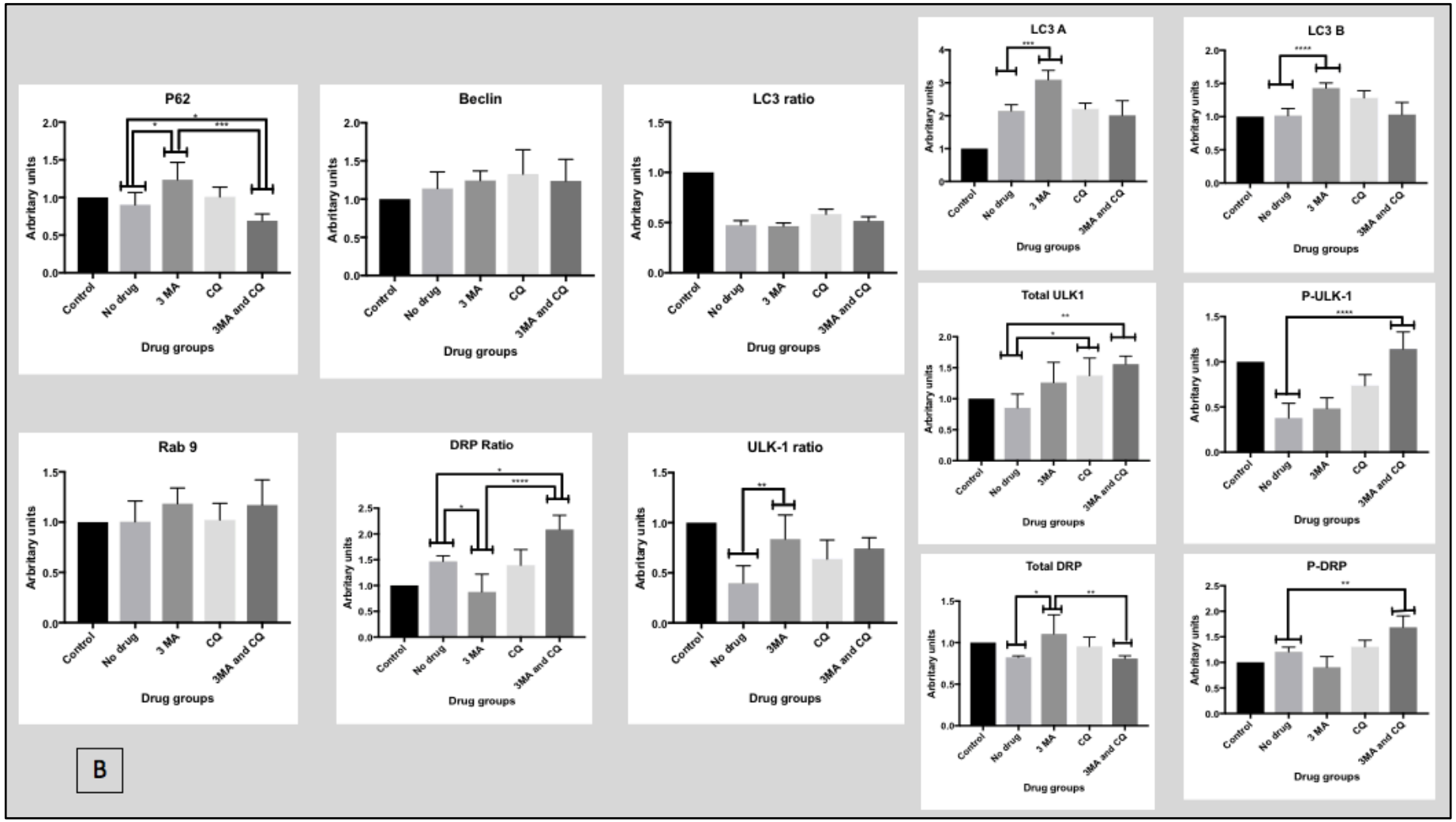


Figure 6.32B: Western blotting data for the 3 Methyl adenine (3MA), with and without Chloroquine (CQ), late reperfusion group
Bar graphs depicting analysed results for p62, Beclin, Rab 9, LC3 A/B, total (T) and phosphorylated (P) ULK1 and DRP1, and the ratio's for LC3, ULK1 and DRP1. Data presented as mean ± SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

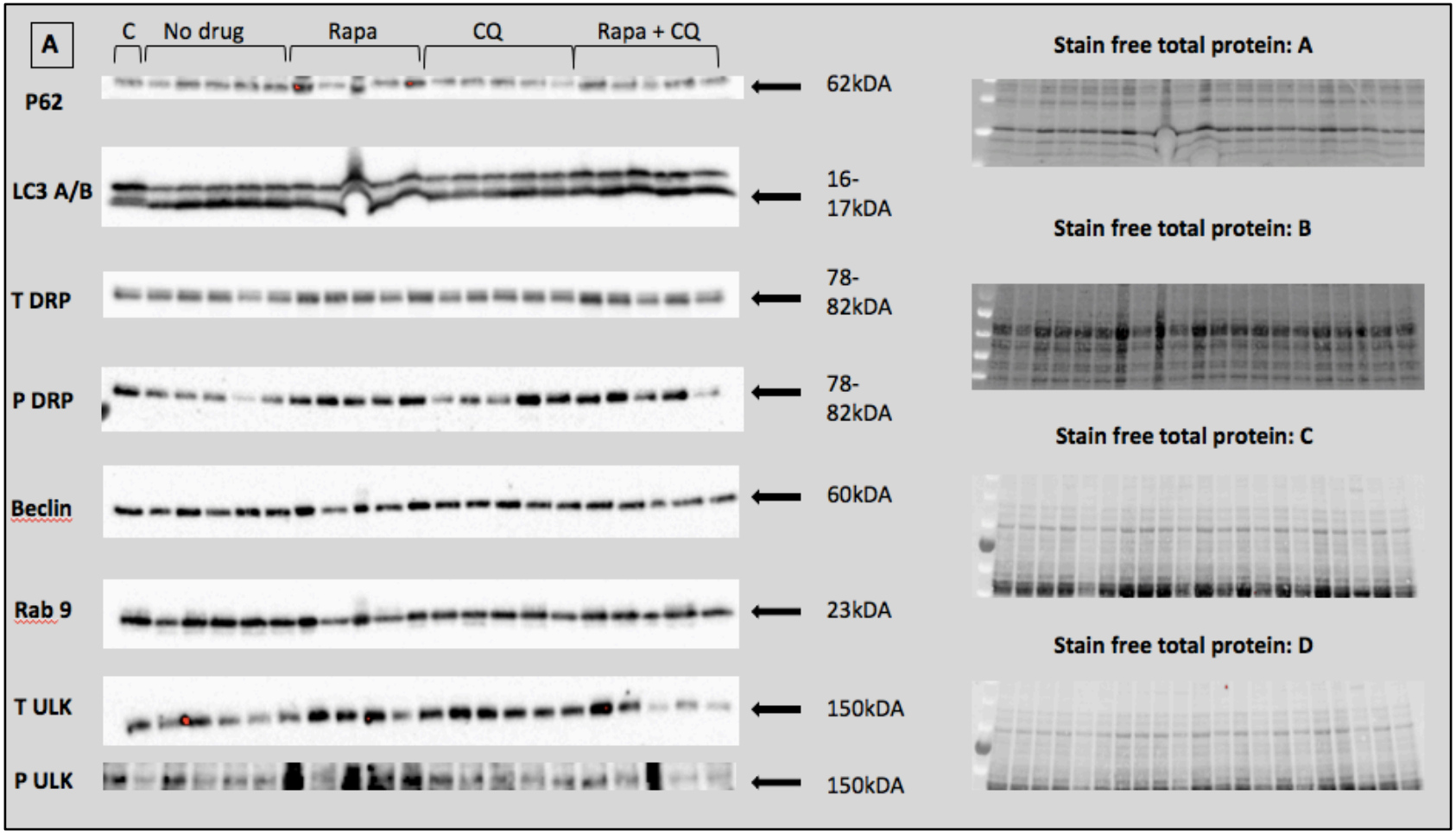


Figure 6.33A: Western blotting data of samples collected during reperfusion after exposure of hearts to 20 min global ischemia and 1 nM Rapamycin (rapa) during late reperfusion ± Chloroquine (CQ) pretreatment. n=5 hearts/group

Western blot chemiluminescent results of P62, Beclin and LC3 A/B, Rab 9, Total (T), phosphorylated (P) ULK1 and DRP 1. Stain-free total protein stain of the membranes A, B, C and D. LC3 from membrane A, Beclin and Rab 9 from membrane B, T ULK, T DRP and p62 from membrane C and P DRP and P ULK from membrane D.

Abbreviations: Refer to Figure 6.30 A.

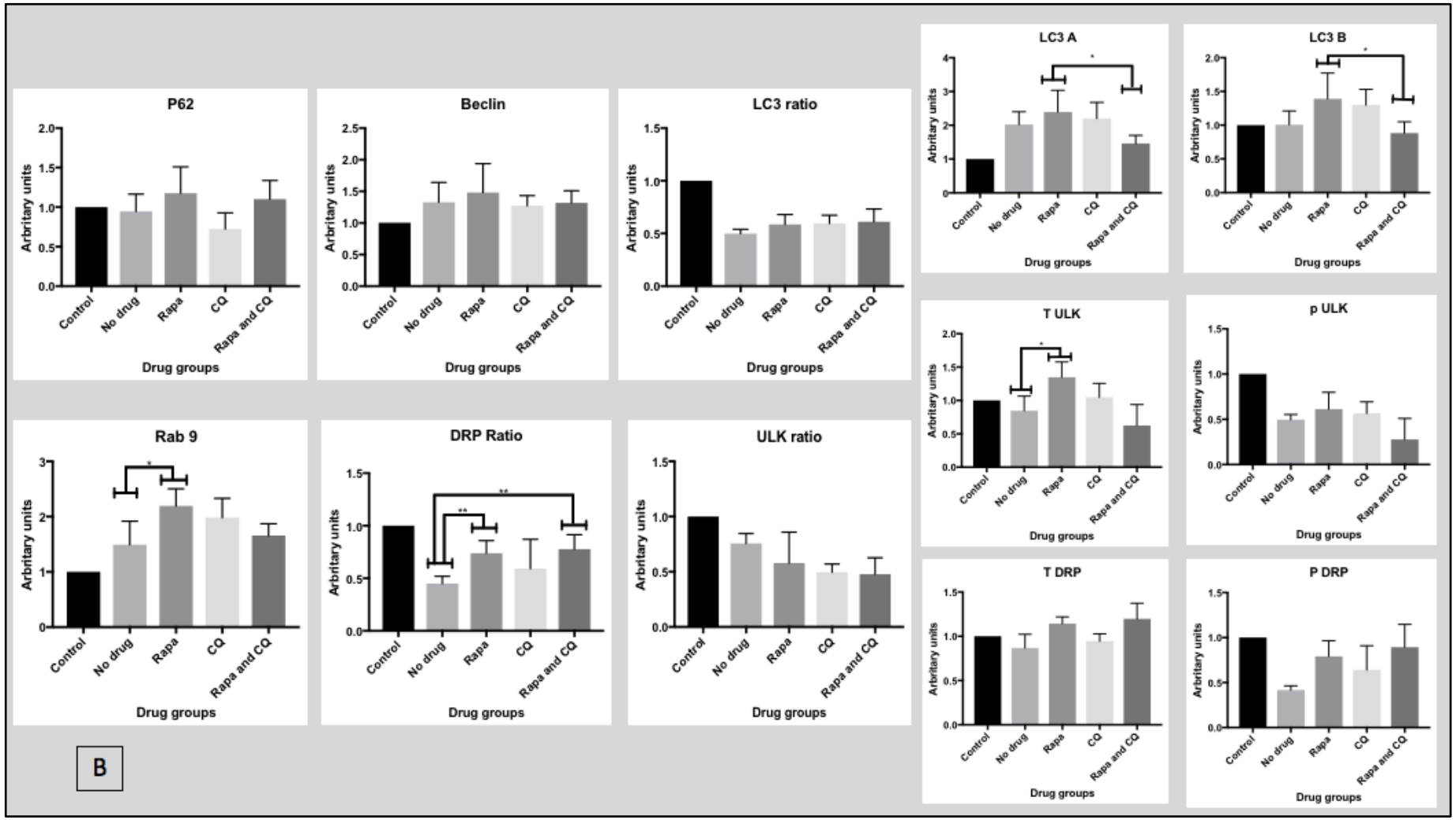


Figure 6.33B: Western blotting data for the 1 nM Rapamycin (Rapa), with and without Chloroquine (CQ), late reperfusion group
Bar graphs depicting analysed results for p62, Beclin, Rab 9, LC3 A/B, total (T) and phosphorylated (P) ULK1 and DRP1, and the ratio's for LC3, ULK1 and DRP1. Data presented as mean ± SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

Table 6.4: Table summarising the net effect of 3 Methyl-adenine addition on steady state autophagy as well as autophagic flux during early and late reperfusion, as compared to untreated hearts

3MA	**Early Reperfusion**		**Late reperfusion**	
Protein	**Steady state**	**Flux**	**Steady state**	**Flux**
P62	-	Decrease	Decrease	Decrease
Beclin	-	-	-	-
LC3 ratio	-	-	-	-
LC3B	-	-	Increase	-
Rab 9	Decrease	Decrease	-	-
ULK1 ratio	-	-	Increase	-
DRP1 ratio	-	-	Increase	Increase

" – " implies that there was no change.

Table 6.5: Table summarising the net effect of 1nM Rapamycin addition on steady state autophagy as well as autophagic flux during early and late reperfusion, as compared to untreated hearts

Rapamycin	**Early Reperfusion**		**Late reperfusion**	
Protein	**Steady state**	**Flux**	**Steady state**	**Flux**
P62	Increase	Decrease	-	-
Beclin	-	Increase	-	-
LC3 ratio	-	-	-	-
LC3B	-	-	-	-
Rab 9	-	-	Increase	-
ULK1 ratio	-	-	-	-
DRP1 ratio	-	Increase	Decrease	Increase

" – " implies that there was no change.

Collectively looking at the summarising tables for the interventional experiments for 3MA (Table 6.4) and Rapamycin (Table 6.5), the following was noteworthy:

- In the early reperfusion 3MA group, except for a decrease in flux as supported by p62, Rab9 was the only protein in which 3MA caused a decrease in steady state and flux.
- When 3MA was administered late and heart free-clamped at 120 min (late reperfusion), p62 showed a consistent decrease in steady state and flux.

- The most important effect following 3MA administration, in both reperfusion groups, is the disappearance of the increase in autophagy with an increase in reperfusion time, as noted in the control experiments.
- Compared to the control results (for 20 min global ischemia), addition of Rapamycin during early reperfusion, caused an increase in steady state autophagy, as indicated by p62. The early Rapamycin group additionally also caused an increase in flux as shown by Beclin and p/tDRP1 ratio, in contrast to the decrease in flux as indicated by p62.
- The very convincing increase in steady state and flux, as seen in the control experiments, according to the LC3 B/A ratio and LC3B, was lost in the late Rapamycin group. The alternative pathway proteins showed an increase in steady state (Rab9) and flux (p/tDRP ratio) autophagy.
- Taking together, Rapamycin administration did not result in the expected increase in autophagy, which was the motivation for repeating the Rapamycin experiments at a higher dosage (Figure 6.34 A and B).

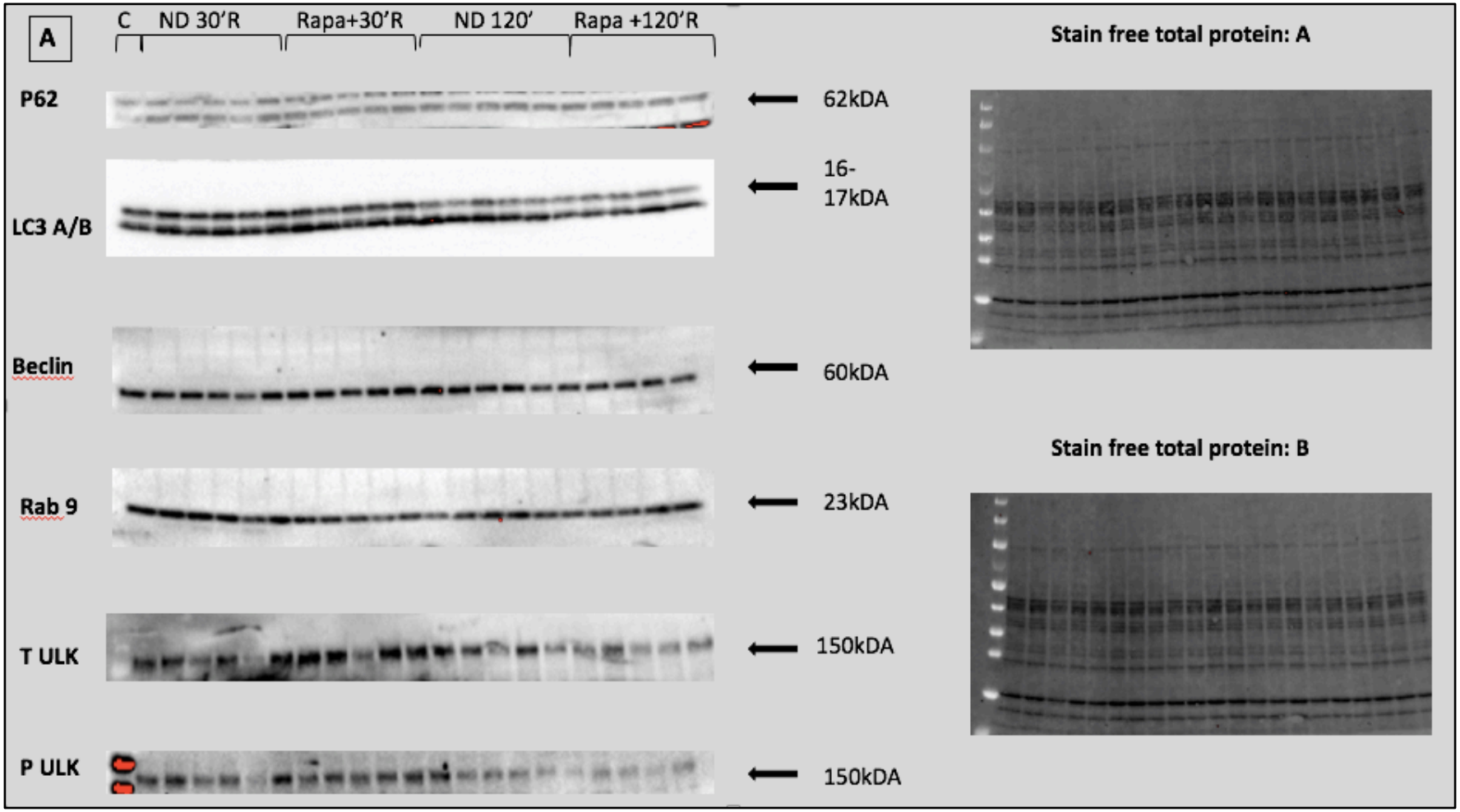


Figure 6.34A: Western blotting data of samples collected during reperfusion after exposure of hearts to 20 min global ischemia and 100 nM Rapamycin during early (30 min) and late (120 min) reperfusion. n=5 hearts/group

Protein chemiluminescent results of P62, Beclin and LC3 A/B, Rab 9, total (T) and phosphorylated (P) ULK1. Stain-free total protein stain of the membranes A and B. LC3, T ULK and P62 from membrane A, Beclin, P ULK and Rab 9 from membrane B. Abbreviations: R: reperfusion, C: control, Rapa: Rapamycin, ND: no drug. kDA: kiloDalton.

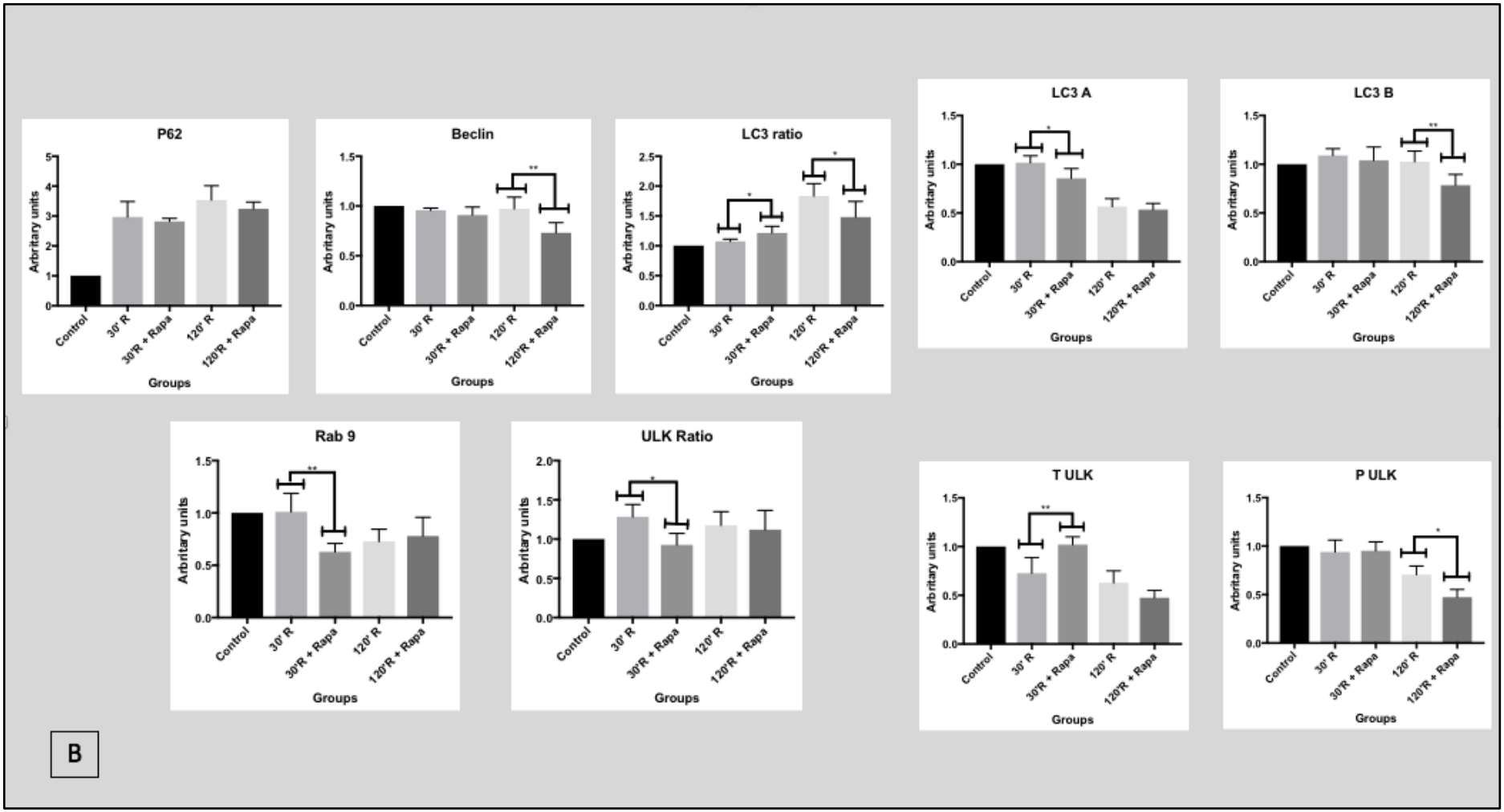


Figure 6.34B: Western blotting data for the 100 nM Rapamycin (Rapa), early (30 min) and late (120 min) reperfusion group

Bar graphs depicting analysed results for p62, Beclin, Rab 9, LC3 A/B, total (T) and phosphorylated (P) ULK1, and the ratio's for LC3, and ULK1. Data presented as mean ± SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

6.3.2.5. 100 nM Rapamycin, early and late reperfusion

In view of the failure to elicit a significant upregulation of autophagic flux with Rapamycin (1 nM) during reperfusion, it was decided to increase the dosage 100 fold (100 nM) for administration during either early or late reperfusion. However, the results obtained in the **100 nM Rapamycin group, for both reperfusion periods** (Figure 6.34 A and B) were also unconvincing for a consistent increase in steady state autophagy.

- Assessing the **LC3 B/A ratio,** a significant increase was found between the no drug and the Rapamycin group **(**1.07±0.02 vs 1.22±0.05, p=0.025), during early reperfusion, suggestion upregulation of autophagy under these conditions.
- This, however, is in contrast to the decrease found (evaluating the same drug groups as above) in **Rab9** levels (1.01±0.09 vs 0.63±0.04, p=0.003) and the **p/tULK1 ratio** (1.29±0.07 vs 0.93±0.07, p=0.0002).
- Two proteins showed an unexpected significant decrease in steady state levels during late reperfusion, namely **Beclin** (no drug: 0.97±0.06 vs rapa: 0.73±0.05, p=0.0143) and the **LC3 B/A ratio** (no drug: 1.83±0.09 vs rapa:1.48±0.12, p=0.047).

This group was not repeated with Chloroquine due to the lack of convincing and consistent results. We elected to repeat the above experiments with an even higher dosage of Rapamycin (250 nM) *and* for a longer period of exposure (30 min instead of 10 min). For the following experiments 250 nM Rapamycin was added to the perfusate during 0-30 min reperfusion (early reperfusion) or 60-90 min reperfusion (late reperfusion)

6.3.2.6. 250 nM Rapamycin, early and late reperfusion with and without Chloroquine.

Following a 250 increase in the original Rapamycin dosage as well as three times the drug exposure, the following were the main findings: (Figure 6.35 A and B)

- Proteins **p62** (no drug: 0.63±0.032 vs rapa: 0.80±0.05, p=0.0234) and **LC3 B/A ratio** (no drug: 1.86±0.10 vs rapa: 2.58±0.22, p=0.0241) both demonstrated a significant increase in autophagic flux during *late* reperfusion.
- The significant reduction in **p62** steady state levels during *early* reperfusion (no drug: 0.65±0.04 vs rapa: 0.49±0.02, p=0.024) suggest upregulation of autophagy. This was however followed by a decrease in flux (0.64±0.04 vs 0.43±0.05, p=0.0159).
- **Rab9**: A significant decrease was found at 30 min reperfusion, between the no drug and the Rapamycin (1.08±0.02 vs 0.95±0.04, p=0.039), as well as the no drug and

Rapamycin and CQ combination (1.08±0.02 vs 0.92±0.05, p=0.0214). A significant decrease between no drug and the Rapamycin CQ combination was also found at 120 min reperfusion (1.06±0.04 vs 0.90±0.02, P=0,0098).

- During early reperfusion **p/tDrp1 ratio** was significantly increased by the combination of Rapamycin and CQ compared to the no drug group (0.86±0.05 vs 1.26±0.01, p=0.0017).

In summary: Table 6.6 gives a graphic representation as well as a summary of the significant changes in the different protein groups for early and late reperfusion (as discussed in statistical detail earlier). The conventional indicators of autophagy namely LC3 B/A ratio, p62 and Beclin, showed that early reperfusion administered 250 nM Rapamycin did not cause a significant increase in autophagic activity, when compared to 1 nM Rapamycin. This will be discussed in Chapter 7. However, following late reperfusion, the increase in Rapamycin dosage to 250 nM resulted in a significant increase in autophagic flux, as assessed by LC3 ratio and p62. The results obtained from Beclin were largely disappointing, with only one significant change (increase in flux) during early reperfusion in the 1 nM Rapamycin group.

p62 as indicator of autophagy supported an expected decrease in steady state and flux (after late reperfusion) and flux alone (following early reperfusion) when 3MA was administered.

The LC3 B/A ratio, a very common autophagic protein marker, exhibited a consistent and predicted increase in steady state and flux during the late reperfusion *control* group. These increases disappeared following 3MA and (unexpectedly) 1 nM Rapamycin administration. In addition this ratio proved to be a more reliable indicator of autophagy than LC3B which only showed a significant difference in the late 3MA group.

Table 6.6: Summary of the significant changes for the individual proteins, in the different drugs groups for both reperfusion intervals

Protein	Early reperfusion								Late reperfusion							
	C	C+ CQ	3MA	3MA+CQ	R	R+CQ	RR	RR+CQ	C	C+CQ	3MA	3MA+CQ	R	R+CQ	RR	RR+CQ
LC3 ratio	x	x	x	x	x	x	x	x	↑	↑	x	x	x	x	x	↑
LC3B	x	↑	x	x	x	x	x	x	↑	↑	↑	x	x	x	x	x
P62	↑	x	x	↓	↑	↓	↑	↓	x	x	↓	↓	x	x	x	↑
Beclin	x	x	x	x	x	↑	x	x	↑	x	x	x	x	x	x	x
ULK1 ratio			x	x	x	x	↓	↓			↑	x	x	x	x	x
DRP1 ratio			x	x	x	↑	x	↑			↑	↑	↓	↑	x	x
Rab9			↓	↓	x	x	↓	↓			x	x	↑	x	x	↓

Abbreviations: C: control, 3MA: 3Methyl-adenine, R: Rapamycin at 1 nM, RR: Rapamycin at 250 nM, CQ: Chloroquine, x: no change, down arrow: significant decrease in autophagic activity when compared to post ischemic/ no drug group, up arrow: significant increase in autophagic activity when compared to post ischemic/ no drug group.

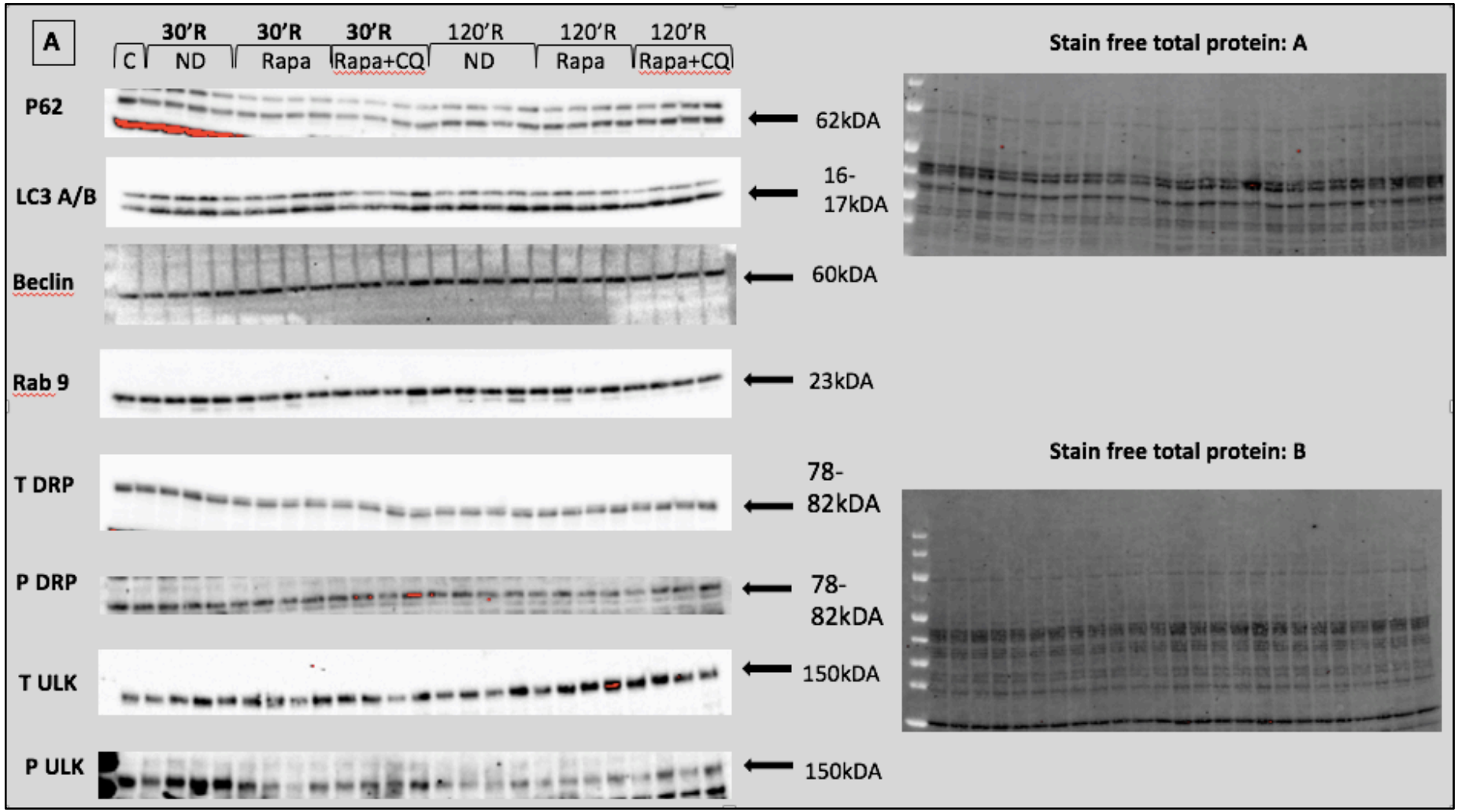


Figure 6.35A: Western blotting data of samples collected during reperfusion after exposure of hearts to 20 min global ischemia and 250 nM Rapamycin (Rapa) during early (30 min) and late (120 min) reperfusion ± chloroquine. n=4 hearts/group

Protein chemiluminescent results of P62, Beclin and LC3 A/B, Rab 9, total (T) and phosphorylated (P) ULK1. Stain-free total protein stain of the membranes A and B are included. P62 is from membrane A and LC3, T ULK, P ULK, Beclin, Rab9 and LC3 are from membrane B. Abbreviations: See Figure 6.34.

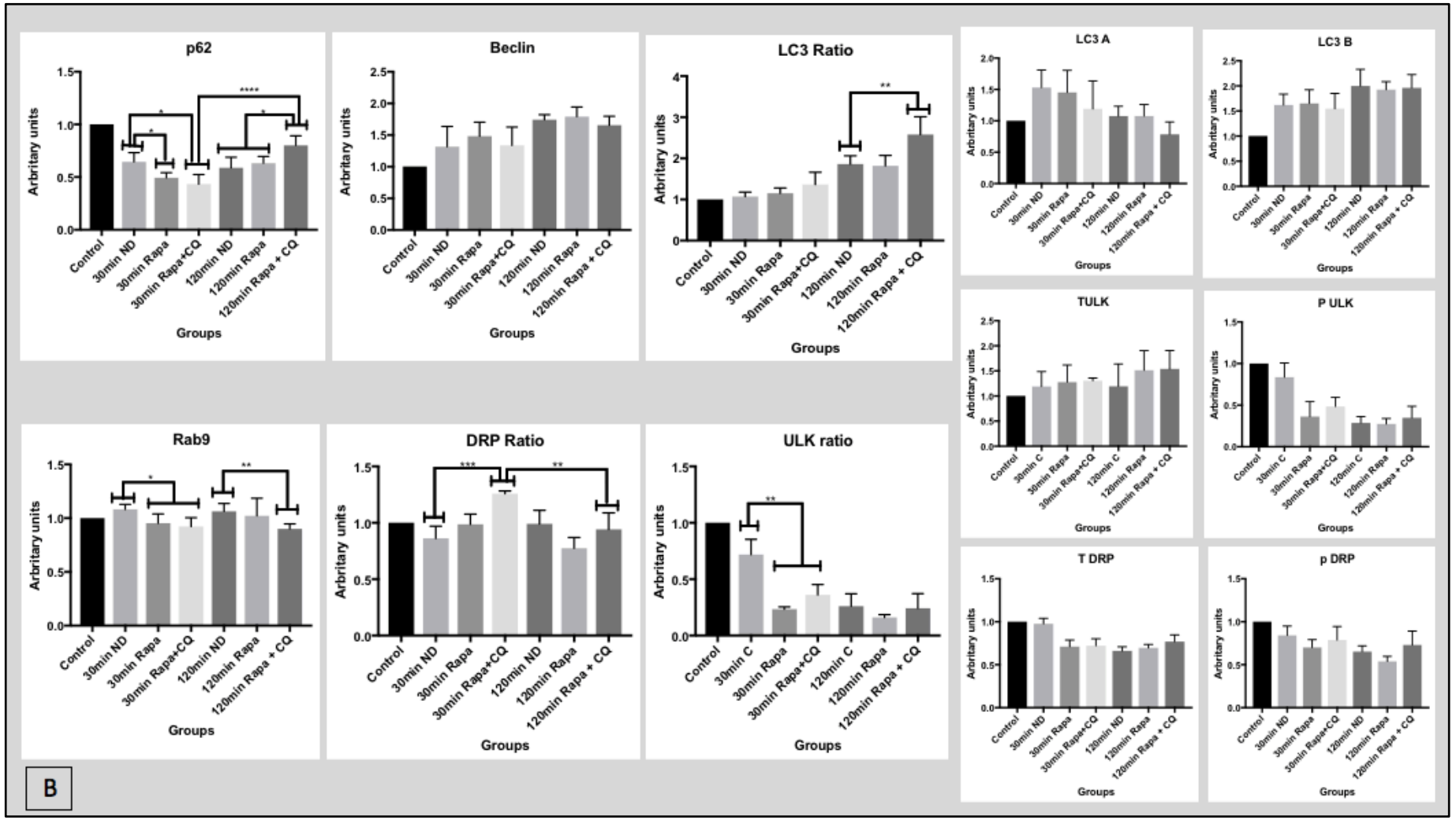


Figure 6.35B: Western blotting data for the 250 nM Rapamycin (Rapa), early (30 min) and late (120 min) reperfusion group, with and without Chloroquine (CQ)

Bar graphs depicting analysed results for p62, Beclin, Rab 9, LC3 A/B, total (T) and phosphorylated (P) ULK1 and DRP1, and the ratio's for LC3, DRP1 and ULK1. Data presented as mean ± SEM (* $p < 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p<0.0001$)

6.4. INFARCT SIZE ANALYSIS DATA

Neither early (% of area at risk: 52.07±2.94) nor late (% of area at risk: 54.71±3.99) reperfusion administered Rapamycin (at 1 nM), influenced the infarct size significantly when compared to the control hearts (% of area at risk: 52.90±5.13) (p=0.894 for early and p=0.798 for late administered Rapamycin). In considering all the infarct size groups together (Figure 6.36 and Table 6.7), CQ pre-treatment, in addition to Rapamycin and the control groups, also did not result in any substantial changes in infarct size (p>0.999 for control vs control + CQ, Rapamycin early vs Rapamycin early + CQ and Rapa late vs Rapamycin late + CQ).

Addition of 3MA to the perfusate during early reperfusion, resulted in a significant decrease in infarct size when compared to the control (% of area at risk: 52.90±5.13 vs 30.08±2.19, p=0.0004), early Rapamycin (% of area at risk: 52.07±2.94 vs 30.08±2.19, p=0.0012), late Rapamycin (% area at risk: 54.71±3.99 vs 30.08±2.19, p=0.0005) and late 3MA (% of are at risk: 50.89±2.76 vs 30.08±2.19, p=0.0015) groups. The addition of CQ in this early 3MA group resulted in an insignificant increase in infarct size (when compared to early-3MA group) when analysed by ANOVA (p=0.463), but a significant increase (% of area at risk: 30.08±2.19 vs 43.42±1.94, p=0.0009) when the two groups were compared with an unpaired Students t test (Figure 6.36). Late reperfusion administered 3MA (% of area at risk: 50.89±2.76) had no pronounced effect on infarct size when compared to the control (% of area at risk: 52.90±5.13, p=0.735) and other groups .

In comparing the areas at risk, no significant differences were found between the groups (p>0.999 for all area at risk group comparisons) (Figure 6.37 and Table 6.7).

Table 6.7: Table summarising the mean (±SEM) of the myocardial infarcts sizes (as a % of the area at risk) and the average area at risk (% of the left ventricle), following 35 minutes of LAD coronary occlusion and 120 minutes reperfusion, for the different drug groups

Groups	Infarct size	Area at risk
Control group	52.90±5.13	39.68±2.08
Control group + CQ	52.79±3.91	40.55±5.13
Rapamycin early R	52.07±2.94	43.56±1.77
Rapamycin late R	54.71±3.99	37.62±7.34
Rapamycin + CQ early R	52.07±2.94	39.79±4.01
Rapamycin + CQ late R	53.02±5.03	37.49±2.75

3MA early R	30.08±2.19	40.28±5.23
3MA late R	50.89±2.76	39.84±1.41
3MA + CQ early R	43.42±1.94	39.05±1.61

Early and late refers to when the drug was administered during reperfusion, early administration took place during 0 - 10 min and late during 50 – 60 min of reperfusion. (n=6-8) Abbreviations: R: reperfusion, 3MA: 3 Methyl-adenine, %: percentage, LAD: left anterior descending and CQ: Chloroquine.

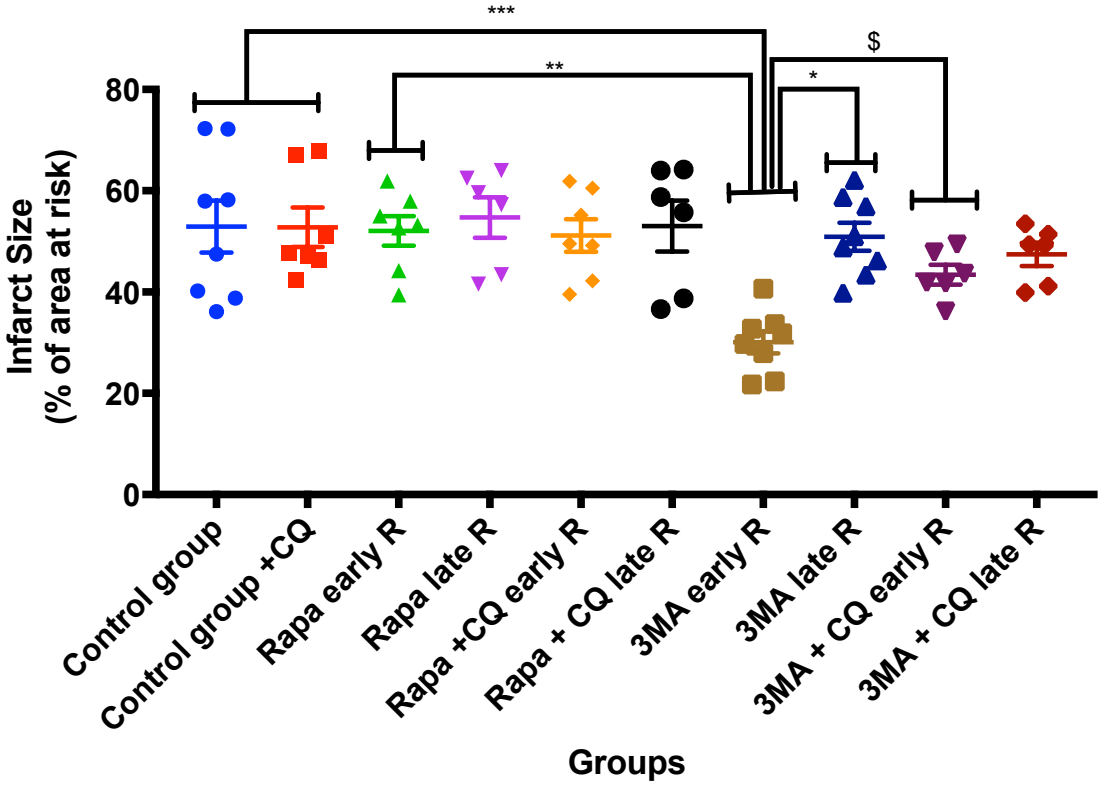


Figure 6.36: Scatter plot with the mean and SEM for the myocardial infarct size following 35 minutes of LAD coronary occlusion and 120 minutes of reperfusion, in the groups as indicated

Early and late refers to when the drug was administered during reperfusion, early administration took place during 0 - 10 min and late during 50 – 60 min of reperfusion. $ indicates the statistical significant difference (p=0.0009) found with separate Student's t test analysis; * p< 0.05, ** p< 0.01, *** p< 0.001 (n=6-8). Abbreviations: R: reperfusion, 3MA: 3 Methyl-adenine, Rapa: 1 nM Rapamycin, CQ: Chloroquine, min: minutes, %: percentage and LAD: left anterior descending.

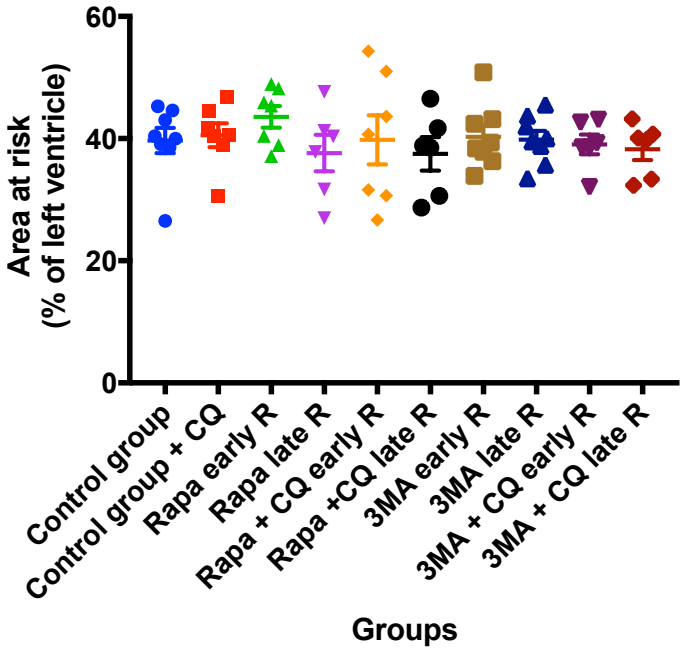


Figure 6.37: Scatter plot with the mean and SEM for the area at risk, following 35 minutes of LAD coronary occlusion and 120 minutes of reperfusion, in the groups as indicated

Early and late refers to when the drug was administered during reperfusion, early administration took place during 0 - 10 min and late during 50 – 60 min of reperfusion. (n=6-8) Abbreviations: R: reperfusion, 3MA: 3 Methyl-adenine, Rapa: 1 nM Rapamycin, CQ: Chloroquine, min: minutes, %: percentage and LAD: left anterior descending.

Extending the exposure time (from 10 min to 30 min) in the **250 nM Rapamycin** group, implied a new perfusion protocol to facilitate drug administration during Langendorff mode (Section 5.12.3). This group can therefore not be compared to the other groups in terms of infarct size. Neither the early reperfusion (% of area at risk: 46.08±2.04, p=0.1009), late reperfusion (% of area at risk: 56.32±2.65, p=0.2751) nor late reperfusion with CQ (% of area at risk: 54.01±5.43, p=0.7126) demonstrated statistically significant differences when compared to the control group (% of area at risk: 52.00±2.64) (Table 6.8 and Figure 6.38). The increase in autophagic flux during late reperfusion, as a result of 250 nM Rapamycin, therefore did not result in a significant change in infarct size.

Table 6.8: Table summarising the mean (±SEM) of the infarcts sizes (as a % of the area at risk) and the average area at risk (% of left ventricle), following 35 min of LAD coronary occlusion and 120 min reperfusion, for the different drug groups

Groups	Infarct size	Area at risk
Control group	52.00±2.64	41.49±1.32
250 nM Rapamycin, early R	46.08±2.04	38.69±2.30
250 nM Rapamycin, late R	56.32±2.65	43.11±1.45

250 nM Rapamycin + CQ late R	54.01±5.43	39.16±3.18

Early and late refers to when the drug was administered during reperfusion, early administration took place during 0 - 30 min and late during 50 – 80 min of reperfusion. (n=6-8). Abbreviations: R: reperfusion and CQ: Chloroquine, %: percentage and LAD: left anterior descending.

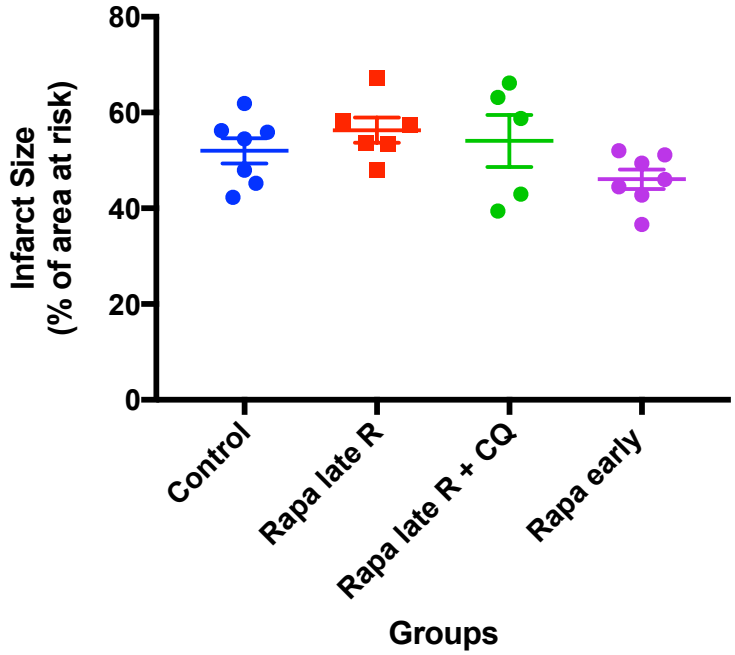


Figure 6.38: Scatter plot with the mean and SEM for the myocardial infarct sizes, following 35 minutes of LAD coronary occlusion and 120 minutes of reperfusion, in the groups as indicated

Early and late refers to when the drug was administered during reperfusion, early administration took place during 0 - 30 min and late during 50 – 80 min of reperfusion. (n=6-8). Abbreviations: R: reperfusion, 3MA: 3 Methyl-adenine, Rapa: *250 nM Rapamycin*, CQ: Chloroquine, %: percentage and LAD: left anterior descending.

In the subsequent chapter (Chapter 7) a discussion will be formulated based on the study's results, highlights, limitations and future directions on this research topic.

CHAPTER 7
DISCUSSION

7.1. INTRODUCTION

The precise role and significance of autophagy during ischemia and reperfusion in the myocardium is still a subject of debate. While it appears that the cardioprotective properties of autophagy during ischemia are context-dependent, the consequences of its manipulation during reperfusion still yield contradicting results (Matsui *et al.*, 2007). (Also refer to Chapter 4)

Ischemic heart disease (IHD) is the leading cause of death worldwide (Finegold, Asaria and Francis, 2013). Each of the treatment modalities in response to myocardial infarction implies the re-establishment of coronary perfusion, and therefore the benefits *and* detrimental effects associated with reperfusion (discussed in Chapter 3). Reperfusion injury (RI) has been estimated to be responsible for up to half of the final infarct size and no effective therapeutic intervention currently exists to entirely prevent the deleterious effects of this phenomenon (Hausenloy and Yellon, 2013; Thapalia, Zhou and Lin, 2014). Comparison of myocardial protection secondary to the manipulation of autophagy during early and late reperfusion has not been studied previously. Thus, the overall aim of the current study was to investigate whether pharmacological manipulation of autophagy could reduce reperfusion injury.

In this chapter, the results presented in Chapter 6 are discussed focusing on answering the following questions:

- Is the pharmacological *induction* of autophagy protective during *early* reperfusion?
- Is the pharmacological *inhibition* of autophagy protective during *late* reperfusion?
- The results are also used to characterize the pattern of autophagic flux during early and late reperfusion in our experimental model, and to evaluate the effect of different periods of ischemia on autophagic steady state and flux during reperfusion.

7.2. EXPERIMENTAL MODEL

Langendorff's classic method for the retrograde perfusion of an isolated mammalian heart (Langendorff, 1895) is commonly used in cardiovascular physiological studies. Despite the enormous contribution that this particular model has made to current knowledge regarding

the function and metabolism of the heart, it also has significant shortcomings. The primary problem with the retrogradely perfused heart is that it does not perform the work of a pump and is therefore often referred to as a "non-working" preparation. Neely *et al*. (1967) developed a modification of the classical method to obtain the so-called isolated perfused "working" heart model (Neely *et al.*, 1967). In this perfusion model two cannulas are being used, one placed in the aorta, and a second one either into the pulmonary vein or in the left atrium. The perfusate is being introduced into the left atrium at 15 cm H2O (the preload) and pumped by the left ventricle against 100 cm H2O (the afterload) (Neely *et al.*, 1967). It is recognized that the pressure in the atrium does not directly equate to preload but it is an acceptable surrogate for preload under these controlled conditions.

This model allows for studies to be done over a wide range of work and pressures. It also allows for the most objective study of mechanical activity, since this heart preparation does not include other tissues, organs or circulatory neuro-hormonal factors which may potentially influence or complicate results, as encountered in an intact animal experimental model (Lateef, Al-Masri and Alyahya, 2015). Thus the rat heart, as an animal model to investigate the effects of ischemia and reperfusion, is ideal since it:

- Mimics various clinical conditions such as ischemia/reperfusion.
- Responds to drugs in a manner that correlates with the human clinical response.
- Reflects adequate precision and accuracy to function as a bioassay.
- Allows for a variety of measured responses.
- Is practical from a time, cost and availability point of view (Curtis, Macleod and Walker, 1987).

Certain advantages and disadvantages are associated with the isolated perfused rat heart (Langendorff in combination with the working heart) model: The advantages include:

- The rat heart has a uniform lack of effective coronary collaterals, which results in reproducible occluded ischaemic zones (Maxwell, Hearse and Yellon, 1987).
- Rats are small, not expensive and readily available.
- Existence of an extensive physiological database for rats.
- The model has a relatively long viable duration, low associated running costs and simple preparation.
- The data collection from the model is reliable in terms of quality and quantity.

- It allows for a variety of measurements without neuro-hormonal or other organ interference.
- Drug effects on the heart can be studied with ease (Curtis, Macleod and Walker, 1987; Lateef, Al-Masri and Alyahya, 2015).

Disadvantages include that:

- The true clinical relevance is often unclear when extrapolating results obtained in rats, and when using an isolated organ (the heart) (Curtis, Macleod and Walker, 1987).
- The rat may require higher drug doses to achieve particular blood concentrations, which is due to the higher rate of drug metabolism in smaller versus larger animals (Curtis, Macleod and Walker, 1987).
- This model, although viable for several hours, must be considered as a "dying system". A 5 – 10% deterioration per hour in contractile and chronotropic function can be expected when using the Langendorff system (Sutherland and Hearse, 2000; Bell, Mocanu and Yellon, 2011).
- The normal physiological conditions are altered, and with time, functional and structural alterations occur, which will eventually lead to myocardial failure.

The maximum possible reperfusion time in the isolated working rat heart perfusion may vary from laboratory to laboratory and depends on the dexterity of the perfusionist, the duration of ischemia, perfusion protocol and drug interventions.

When considering the above advantages and disadvantages, it is clear why the rat heart Langendorff system, in combination with working heart variations, is the specie and the model of choice for many investigators (Bell, Mocanu and Yellon, 2011; Lateef, Al-Masri and Alyahya, 2015). Its reliability, reproducibility and ease of experimentation are important advantages. The main limitations - clinical relevance and the limited duration of ischemia and reperfusion in the ex vivo model should however also be considered in the interpretation of the results.

7.3. WORKING HEART

7.3.1. Control experiments

The main findings in the *control* experiments include (Table 6.1 and Figures 6.2 - 6.5):

- A significant decrease in global myocardial function (as assessed by work performance (Wtot) and cardiac output (CO)) during 30 min and 70 min reperfusion after both intervals of ischemia (of 15 and 20 min).
- Twenty min of global ischemia was responsible for a statistically more significant reduction in global myocardial function than 15 min of global ischemia, as measured at both early and late reperfusion intervals.
- An increase in reperfusion time did not cause a further reduction in function as illustrated by the absence of a significant difference in CO and Wtot between 30 min and 70 min reperfusion.

According to the protocols used in our study, seventy minutes was the latest reperfusion period opportunity during which working heart data could be collected. A longer reperfusion time may have allowed us to demonstrate a decrease in global myocardial function with an increase in reperfusion time. However, the possible decline of the model during prolonged reperfusion, may lead to inaccuracies in the interpretation of the data. Our pre-ischemic CO and Wtot values however correlate well with values obtained previously in our laboratory (Lochner *et al.*, 1999, 2009)

The administration of Chloroquine (CQ) (by intra-peritoneal injection of the drug 1h prior to heart isolation) in the control experiments did not cause a deviation from the previously mentioned *pattern* of results in the control experiments. Compared to pre-ischemia, a significant decrease in CO and Wtot occurred at both reperfusion intervals, with the results again being more pronounced after 20 min (compared with 15 min) of global ischemia. (Table 6.2 and Figures 6.5 to 6.7)

There was however one subgroup in which CQ pretreatment caused a significant difference in global myocardial function: CQ caused a decrease in function following 20 min of global ischemia and 30 min of reperfusion. (Figures 6.10 and 6.12) Thus pretreatment with Chloroquine was therefore not associated with a *consistent* significant change in function in our experimental model and as yet we do not have an explanation for these divergent results. In the subsequent interventional experiments, CQ pretreatment had no significant effects on myocardial function during pre-ischemia and after 30 min or 70 min of reperfusion. (Refer to the next section, 7.3.2) With the exception of the above-mentioned group, all our control and interventional experimental groups indicated that CQ did not affect global myocardial function.

The ample existing literature supporting the cardiotoxic as well as cardioprotective effects of Chloroquine on the heart will be discussed in section 7.5.3.

7.3.2. Interventional experiments

The most pronounced changes in autophagy were found following a longer ischemic (20 min versus 15 min), early reperfusion (30 min vs 10 min) and late reperfusion (120 min vs 90 min and 60 min) duration. During the interventional experiments 20 min of global ischemia was therefore induced throughout, early and late reperfusion were defined as 30 and 120 min respectively and drugs were added aiming at the manipulation of autophagy.

In evaluating the effects of 3 Methyl-adenine (3MA) (Figure 6.14), 1 nM Rapamycin (Figures 6.16) and 250 nM Rapamycin (Figure 6.22), the following can be stated:

- These two drugs did not change the pattern of the functional myocardial depression at 30 and 70 min reperfusion as was seen in the control experiments, after 20 min of global ischemia.
- The changes recorded in the presence of 3MA and Rapamycin (1 and 250 nM), were of similar magnitude (CO ($p<0.001$) and Wtot ($p<0.001$)) as was noted in the *control* experiments.
- As referred to earlier, treatment with CQ in the interventional studies did not cause a significant change in Wtot (Figures 16.18&19) or CO (Figures 16.20&21).
- No statistically significant difference (in either CO or Wtot) could be detected in comparing the groups (control, 3MA and 1 nM Rapamycin, all with and without Chloroquine) for the *same reperfusion period*. (Figures 6.18 – 6.21) (250 nM Rapamycin group could not be included in this comparison since we had to use a different perfusion protocol to allow for the extended duration of exposure as discussed in Chapter 5, section 5.12.)

In the context of this study, the lack of significant differences in the myocardial work performance during reperfusion after 3MA and Rapamycin (1 and 250 nM) administration, may be attributed to the following possibilities:

1. Autophagy was not manipulated effectively by the drugs.
2. The degree of autophagic manipulation was not sufficient to affect functional recovery during reperfusion.
3. A change in autophagic activity may not change global myocardial function i.e. it did

not play a significant role in the pathophysiology.

4. The time constraints as a result of our model did not allow us to register changes in global function (longer allowed reperfusion may demonstrate a change in function).
5. The myocardial suppressent due to stunning (see Section 3.2.2) following ischemia, could override the drugs effects.

The ability of the drugs to manipulate autophagy (sufficiently) will be discussed in section 7.4.

The answer to the question whether autophagic manipulation will influence global myocardial function, is not clear in the context of this study.

Normally, as discussed in the literature review (Chapter 4), autophagy functions as a pro-survival pathway during cellular stress by removing protein aggregates and damaged organelles. During severe stress situations, autophagy may be upregulated excessively and therefore lead to autophagic induced cell death (De Meyer and Martinet, 2009). An optimal degree of autophagic activity therefore seems to exists, which is critical to the maintenance of cardiovascular homeostasis and function (Lavandero *et al.*, 2015). A deviation from the optimal window of autophagy *might* influence global myocardial function secondary to autophagic cell death (excessive induction) or reduction in energy supply following a decrease in protein removal (autophagy inhibition).

The ultimate autophagy level, in our model, will depend on the sum of the baseline autophagy activity, the degree of upregulation secondary to ischemia and reperfusion and the manipulation thereof as a consequence to drug administration. The autophagic process consists of a sequence of interrelated steps: trigger, autophagosome formation, cargo recognition, autophagosome-lysosome fusion and breakdown of the cargo followed by release of the degradation products back into the cytosol. In view of these processes, there is the probability that it will not be able to show an *immediate* effect. Therefore, irrespective of whether the autophagic activity was changed sufficiently to influence global myocardial function, the clinical evidence may not be evident in the time course allowed by our experimental model. Even though, to the best of my knowledge, there is no available research available describing the time course of autophagy in a model similar to ours, this is likely to be an important contributing factor to the lack in significant changes observed.

In summary, the results obtained in our working heart perfusion model demonstrate the expected decrease in global myocardial function following global ischemia. This decrease

was more pronounced following an increase in ischemic time (from 15 to 20 min). Interestingly, our data did not show a decrease in function with increased reperfusion time, indicating that our model was stable over the long experimental period. The administration of CQ (and therefore the inhibition of autophagosome and lysosome fusion) was not associated with a statistically significant change in myocardial function in either the experimental groups. There was only one control group (20 min of global ischemia followed by 30 min reperfusion) in which Chloroquine pretreatment resulted in a significant decrease in global myocardial function, an observation for which we do not have an explanation as yet.

In addition, neither 3MA, 1 nM or 250 nM Rapamycin administration had significant effects on functional recovery during reperfusion when compared with the controls.

7.4. SIGNALING PROTEINS WITH INTERPRETATION IN TERMS OF AUTOPHAGY MANIPULATION

The perfect autophagy assessment tool unfortunately does not exist. The quest for better, more accurate and reliable assays to evaluate autophagic steady state and flux continues. There still is significant controversy with regards to the use and the interpretation of most of the markers. This state of affairs must be taken into account when interpreting the results (Klionsky *et al.*, 2016).

7.4.1. LC3B/ LC3A (LC3 B/A) ratio

LC3 (Atg8) is the most well-known and widely monitored autophagy-related protein, (Klionsky *et al.*, 2012) and the only protein marker which is reliably associated with completed autophagosomes.

Endogenous LC3 is detected as two bands following western blotting: LC3A (molecular mass around 16-18 kD), which is cytosolic, and the other LC3B (14-16 kD), which is conjugated with phosphatidylethanolamine (PE) and mainly present on autophagosomes, but also to a lesser extent, on isolation membranes (phagophores). The conversion of LC3A to LC3B represents PE-conjugation, (Mizushima and Yoshimori, 2007) and it is because of the close correlation between LC3B and autophagosomes that LC3B is used as an indicator of the number of autophagosomes (Klionsky, Cuervo and Seglen, 2007).

There are a number of **caveats** when using LC3 to interpret autophagy activity in mammalian cells:

- The LC3A to LC3B conversion pattern depends on the type of tissue/cell and the degree of stress it was subjected to (and therefore also the treatment used to induce autophagy). LC3A and LC3B levels are not very sensitive, and may therefore not reflect a *slight* induction of autophagy (e.g., by Rapamycin) (Klionsky *et al.*, 2012).
- LC3B itself is also degraded by autophagy and therefore does not always accurately reflect total autophagic flux at a certain point in time. LC3 immunoblotting may therefore sometimes be inappropriately underestimated (Mizushima and Yoshimori, 2007).

Another important issue concerns **which** of the two proteins should **be quantified**, LC3A, LC3B or the LC3B /LC3A ratio? Another option would be to compare LC3B to β-actin or tubulin (or other appropriate "housekeeping" proteins), an approach which is preferred to the LC3B /LC3A ratio. If the Western blot displays faint LC3A bands, the amount of LC3A may be variable and the use of LC3A, as a denominator for quantification of LC3B/LC3A ratio, unreliable (Jiang and Mizushima, 2015; Yoshii and Mizushima, 2017). Also, if LC3A is very high in comparison to LC3B it can be difficult to quantify subtle changes in LC3B relative to LC3A. On the other hand, by completely ignoring the LC3A levels, the overall picture of the cellular autophagic response may be overlooked (Klionsky *et al.*, 2012). Some antibodies may also have a greater affinity for LC3B and the LC3A/LC3B signal ratio may then not accurately reflect the cytosolic to membrane-bound LC3 ratio (Mizushima and Yoshimori, 2007).

In conclusion, the amount of LC3B, LC3B/LC3A and even LC3B/ (LC3A + LC3B) ratio is currently being accepted, with the changes in LC3B viewed as the more accurate approach (Mizushima, 2007; Mizushima and Yoshimori, 2007; Klionsky *et al.*, 2012; Yoshii and Mizushima, 2017). In the present study both the bands representing LC3A and B were consistently clear and reliable in our western blots (with LC3A not being disproportionally dark or faint compared to LC3B), we therefore elected to make use of LC3B as well as the LC3 B/A ratio.

LC3B (and other autophagic protein markers) at a given time point does not necessarily reflect the autophagic activity. This is because not only autophagy activation but also inhibition of autophagosome degradation greatly increase and influence the amount of LC3B (Yoshii and Mizushima, 2017). To determine a true change in autophagic activity, and therefore **autophagic flux (functional autophagy)**, the differences in the amount of LC3B in the presence versus the absence of lysosomal protease inhibitors (for example

Chloroquine or Bafilomycin A_1), have to be determined (Mizushima and Yoshimori, 2007). An increase in autophagic protein activity observed in the presence of Chloroquine, when compared to without, is indicative of an increase in autophagic flux (Klionsky *et al.*, 2012).

Table 7.1: Summary of the significant changes for LC3 B/A ratio and LC3B, in the different drug groups, during early and late reperfusion

Protein	Early reperfusion								Late reperfusion							
	C	C +CQ	3MA	3MA +CQ	R	R+CQ	RR	RR+CQ	C	C +CQ	3MA	3MA +CQ	R	R+CQ	RR	RR+CQ
LC3 B/A ratio	x	x	x	x	x	x	x	x	↑	↑	x	x	x	x	x	↑
LC3B	x	↑	x	x	x	x	x	x	↑	↑	↑	x	x	x	x	x

Abbreviations: LC3 B/A ratio: LC3B/LC3A, C: Control, 3MA: 3 Methyl-adenine, R: Rapamycin at 1nM, RR: Rapamycin at 250 nM for extended exposure, CQ: chloroquine, x: no change, down arrow: significant decrease when compared to post ischemic/ no drug group, up arrow: significant increase when compared to post schemic/ no drug group.

Table 7.1 summarises the LC3 B/A ratio and LC3B data for the control and interventional experiments, for both early and late reperfusion. LC3 B/A ratio (in the absence and presence of the manipulating drugs) did not show any significant changes during the early reperfusion period. LC3B activity indicated a significant increase in autophagic flux in the control experiments, this increase was however abolished in all the early interventional groups.

Following late reperfusion, the LC3 B/A ratio and LC3B results were as predicted for the control experiments, demonstrating an increase in steady state and autophagic flux upon prolongation of the reperfusion time. When comparing the control with the 3MA (autophagic inhibition) group, although 3MA did not cause a significant reduction in autophagy (in comparison with the no drug group), it eliminated the convincing increase in flux observed in the control group. The increase in autophagy as a result of 1nM Rapamycin was too little to result in a significant increase in steady state or flux, the fact that it however also abolished the increase in autophagy with an increase in reperfusion (as seen in the control group) was unexpected. In the 250 nM Rapamycin group a significant increase in the LC3 B/A ratio was detected suggesting an upregulation of autophagic flux at this stage (which is supported by the results of p62, as will be referred to later).

7.4.2. p62

SQSTM1 (sequestosome 1)/p62 (MW 62 kDa), is an adaptor protein that is consumed during autophagy (Ma, Liu, Foyil, Godar, Weinheimer and Diwan, 2012) by being incorporated into the completed autophagosome followed by autolysosomal degradation. It may therefore reflect the degree of autophagic degradation (Klionsky *et al.*, 2012).

According to Gottlieb and coworkers, evaluation of p62 activity can be regarded as a reliable alternative method for detecting autophagic steady state and flux (Gottlieb *et al.*, 2015). In **steady state** autophagy the level of p62 will **decrease** during autophagic induction and **increase** during autophagic suppression, thus an inverse relationship exists between autophagic steady state and p62 levels (Mizushima and Yoshimori, 2007; Nakai *et al.*, 2007; Bartlett *et al.*, 2011). The interpretation during **flux** experiments is however the same for all the proteins, namely an increase in p62, in the presence of chloroquine, can be interpreted as an **increase** in activity and therefore autophagic flux.

There are a number of factors that may potentially influence p62 levels, which include:

- A change in autophagic activity.
- The transcriptional regulation of p62: p62 mRNA levels are upregulated in muscles following exercise, and further accentuated upon starvation. This could mask a decrease in p62 during autophagy (Sanchez *et al.*, 2014). It is therefore advisable to take note of p62 mRNA expression when interpreting p62 data (Yoshii and Mizushima, 2017).
- p62 also participates in proteasomal degradation and may increase with proteasome inhibition (Bardag-Gorce *et al.*, 2005).
- It acts as a substrate for caspase 6 and caspase 8 (enzymes involved in apoptosis) which, in turn, may affect results regarding cell death and autophagy (Norman, Cohen and Bampton, 2010).
- The time course of events also needs to be taken into account: an alteration in p62 levels may not immediately be evident following chemical changes/manipulation. While LC3 changes may be rapid, the clearance of p62 may require more time. Ideally P62 levels are supposed to be monitored at the same time as LC3 but should also be repeated at a later stage to be able to determine the maximal impact of a particular intervention (Klionsky *et al.*, 2012).

- p62 becomes Triton X-100-insoluble in the presence of protein aggregates and when autophagy is inhibited. p62 activity may therefore be lower when using Triton-X-100 and may be context-dependent (Triton-X-100 is part of the lysis buffer used in making lysates for western blot analysis) (Fujita *et al.*, 2011). If possible, p62 levels should be measured in both Triton X-100-soluble and -insoluble fractions (Klionsky *et al.*, 2012; Yoshii and Mizushima, 2017).

From the above list it is clear that there are autophagy-independent factors influencing the p62 expression level (Mizushima and Yoshimori, 2007).

Use of p62 data in isolation may thus not be sufficient to estimate the autophagic activity. A clear (and real-time) correlation between increases in LC3B and decreases in p62 can also not be expected under all experimental circumstances. Although p62 analysis is a very popular protein in the assessment of autophagy, it is recommended that it is used only in combination with other proteins (Mizushima and Yoshimori, 2007; Klionsky *et al.*, 2012).

Table 7.2: Summary of the significant changes for p62, in the different drug groups, during early and late reperfusion

Protein	Early reperfusion								Late reperfusion							
	C	C+ CQ	3MA	3MA +CQ	R	R+C Q	R R	RR+C Q	C	C+ CQ	3M A	3MA +CQ	R	R+C Q	R R	RR+ CQ
P62	↑	x	x	↓	↑	↓	↑	↓	x	x	↓	↓	x	x	x	↑

Abbreviations: C: Control, 3MA: 3Methyl-adenine, R: Rapamycin at 1 nM, RR: Rapamycin at 250 nM for extended exposure, CQ: chloroquine, x: no change, down arrow: significant decrease in autopgaic activity when compared to post ischemic/ no drug group, up arrow: significant increase in autophagic activity when compared to post ischemic/ no drug group.

The fact that we did not find the increase in steady state and flux in the control experiments, as supported by LC3B and LC3 B/A ratio, may be attributed to the expected time lapse between the onset of LC3 and p62 events. Our model unfortunately did not allow re-evaluation of p62 levels at a later stage as this would require an increase in reperfusion time, which theoretically could influence autophagic activity.

P62 results following 3MA administration was as predicted, namely a reduction in flux following both early and later reperfusion.

The p62 results obtained for 1 nM and 250 nM Rapamycin during early reperfusion (increase in steady state and a decrease in flux) are unexpected and not supported by other proteins, such as LC3. In fact, both Rapamycin concentrations appear to elicit a greater reduction in autophagy than 3MA administration during early reperfusion. If this was purely a Rapamycin drug related effect, the same would have been expected during late reperfusion. However, it is more likely to be due to the delay in p62 response in combination with the time lapse between drug administration and freeze-clamping of the hearts (Figure 7.1). During early reperfusion, the Rapamycin was administered from 0-10 min (1 nM) or 0-30 min (250 nM), and freeze-clamped at 30 min. In the late reperfusion group drug administration occurred from 50-60 min (1 nM) and 50-80 min (250 nM), and the hearts were freeze-clamped at 120 minutes. p62 levels (known to change at a slower rate than LC3) thus had the opportunity to respond in the late reperfusion group. Should this be accountable for the difference between the two reperfusion groups, it can be deduced that p62 needs a longer period to yield reliable results.

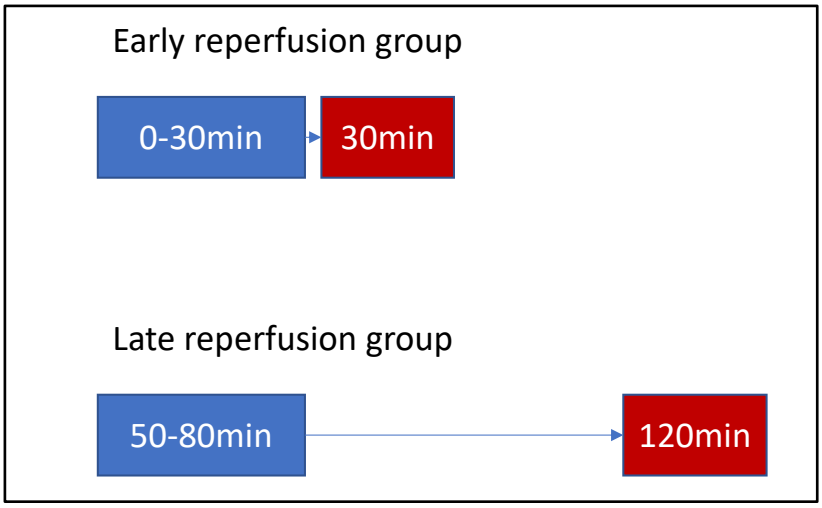


Figure 7.1: Graphic representation of the time lapse between 250nM Rapamycin administration and freeze-clamping the heart during early and late reperfusion

The blue blocks represent drug administration and the red freeze-clamping. Abbreviations: Min: minutes.

Interestingly, the late-250 nM Rapamycin group was the only experimental condition where the expected upregulation of autophagic flux could be seen when evaluating p62 activity. This also correlates well with the LC3 B/A ratio results (see Section 7.4.1.). The fact that there was a significant increase in flux, but not in steady state (in both the mentioned proteins) may be ascribed to the fact that the induction of autophagy (even with the higher dosage and longer period of administration) was still relatively mild.

It is also worth mentioning that two bands were observed in the majority of p62 blots, this was also detected previously (Pankiv *et al.*, 2007; Klionsky, 2009; Bartlett *et al.*, 2011). The lower band may represent a splicing variant of p62 or a partially cleaved product (Klionsky, 2009).

The second band was not visible if the gels were run for a shorter duration during western blotting. In the majority of instances the gels were however ran for as long as possible – this allowed sufficient separation to allow visualization of both ULK1 and DRP on one membrane. During these instances, the protein marker was used to identify the “true” p62’s position. Analysis of both bands was done for a couple of the control results – no differences in results were obtained.

7.4.3. Beclin1

Beclin1 (MW 60 kDa), the mammalian homologue of yeast Atg6, plays a central role in autophagy, and is used by many researchers to monitor autophagic activity (Klionsky *et al.*, 2012). It is also important in the cross-regulation between autophagy and apoptosis, and

modulators of autophagy as well as apoptosis may influence its activity (Kang *et al.*, 2011; Xie, Kang and Tang, 2016). (Refer to Chapters 1 and 4 for additional detail.)

It is important to be aware of the fact that certain forms of macroautophagy are induced in a Beclin1-independent manner. Several reports of Beclin1-independent autophagy pathways have been made recently and these pathways are not blocked by phosphatidylinositol 3-kinase inhibitors (like 3MA) (Chu, Zhu and Dagda, 2007; H. Zhu *et al.*, 2007; F Scarlatti *et al.*, 2008; Tian *et al.*, 2010; Wong *et al.*, 2010; Seo *et al.*, 2011).

During this alternative/ non-canonical/ beclin-independent autophagy (refer to Section1.5, alternative pathways), the formation of the double-membrane-bound autophagosome does not require the normal hierarchical intervention of all the autophagy related gene proteins. It also does not have to elongate from a single membrane source (Codogno, Mehrpour and Proikas-Cezanne, 2011).

The relevance of Beclin-independent pathways was eloquently illustrated by Li *et al*. in 2013. It is well known that both starvation and Rapamycin can induce beclin1-dependent autophagy. Li *et al.* (2013) analysed the consequences of Beclin1 knockdown for LAP (LC3-associated phagocytosis) in cells that had been either starved or treated with Rapamycin. They found that both Rapamycin and starvation treatment enhanced LAP, but that the Rapamycin response is Beclin1 independent whereas the starvation response is Beclin1 dependent (Li *et al.*, 2013). Beclin1 can thus be used to tract conventional autophagic induction, but changes will be absent in Beclin independent autophagy.

For the above mentioned reasons, Klionsky *et al*. (2012) caution against the use of changes in Beclin1 only as a marker of autophagy induction (Klionsky *et al.*, 2012).

Table 7.3: Summary of the significant changes for Beclin1, in the different drug groups, during early and late reperfusion

Protein	Early reperfusion								Late reperfusion							
	C	C +CQ	3MA	3MA +CQ	R	R+CQ	RR	RR +CQ	C	C +CQ	3MA	3MA +CQ	R	R +CQ	R R	RR +CQ
Beclin1	x	x	x	x	x	↑	x	x	↑	x	x	x	x	x	x	x

Abbreviations: C: Control, 3MA: 3Methyl-adenine, R: Rapamycin at 1 nM, RR: Rapamycin at 250 nM for extended exposure, CQ: chloroquine, x: no change, down arrow: significant decrease when compared to post ischemic/ no drug group, up arrow: significant increase when compared to post ischemic/ no drug group.

In the control experiments, Beclin1 activity implied a statistically significant increase in steady state autophagy at 120 min reperfusion (following 20 min of ischemia) (Table 7.3). This however was not evident after early (30 min) reperfusion, and also did not translate into an increase in flux during late reperfusion. This increase was expected considering that ischemia/reperfusion of an in vivo mouse heart, showed that this intervention stimulates autophagy through Beclin 1–dependent mechanisms only (refer to Chapter 4, section 4.4.2.) (Matsui *et al.*, 2007).

During the interventional experiments the Beclin1 results were largely disappointing. There was only one group (early reperfusion, 1 nM Rapa + CQ) that demonstrated a significant change (an increase in flux).

The (lack of) changes in Beclin1 activity may be secondary to:

- Insufficient drug effects.
- Unexpected drug effects, refer to section 7.5.
- The fact that the changes in autophagy occurred via a Beclin1-*independent* pathway.
- The cross-talk between apoptosis and autophagy.

7.4.4. Rab9

The alternative, Atg5/Atg7 independent, autophagy pathway involving Rab9 (MW 23 kDA), is described in Chapter 1, section 1.5. When interpreting cytosolic Rab9 levels, the following are important:

- The alternative pathway is controlled by Ulk1 protein and not by Atg5/Atg7.

- The main trigger for the alternative autophagy pathway is stress and not Rapamycin and nutrient deprivation (as for the conventional pathway).
- LC3A to B alteration does not occur (Shimizu, Arakawa and Nishida, 2010).

In assessing the Rab9 results for the interventional experiments (Table 7.4), an increase in Rab9 associated with Rapamycin administration was therefore not expected. Indeed, a significant increase in Rab9 was found in only one (late reperfusion, 1 nM Rapamycin) of the eight Rapamycin protocol groups. As yet we do not have an explanation for this phenomenon.

The reduction observed in steady state and flux in the early reperfusion of 3MA and 250 nM Rapamycin groups, as well as the decrease in flux in the late reperfusion with 250 nM Rapamycin treatment *may* therefore be attributed to an inhibition of the alternative pathway of autophagy. This conclusion is based on the fact that Rab9 knockdown does not affect macroautophagy (Hirota *et al.*, 2015) and that Rab9 is not required in conventional autophagy (Shimizu, Arakawa and Nishida, 2010).

Table 7.4: Summary of the significant changes for Rab9, in the different drug groups, during early and late reperfusion

Protein	Early reperfusion						Late reperfusion					
	3MA	3MA+CQ	R	R+CQ	RR	RR+CQ	3MA	3MA+CQ	R	R+CQ	RR	RR+CQ
Rab9	↓	↓	x	x	↓	↓	x	x	↑	x	x	↓

Abbreviations: 3MA: 3Methyl-adenine, R: Rapamycin at 1 nM, RR: Rapamycin at 250 nM for extended exposure, CQ: chloroquine, x: no change, down arrow: significant decrease when compared to post ischemic/ no drug group, up arrow: significant increase when compared to post ischemic/ no drug group.

These results are difficult to interpret. Although Rapamycin does not induce the alternative pathway, the results obtained in the present study suggest that the higher concentration Rapamycin inhibits the steady state and flux of the alternative autophagy pathway. The results also suggest that 3MA inhibits both the conventional as well as the alternative autophagic route following early reperfusion.

To the best of my knowledge no literature exists regarding the effects of 3MA or Rapamycin on the alternative autophagic route. It is also noteworthy that the statistically significant reduction in Rab9 levels (with both 3MA and 250 nM Rapamycin) was completely (for 3MA) and partially (for 250 nM) lost with an increase in reperfusion time.

7.4.5. Phosphorylated/total DRP1 ratio (p/tDRP1)

Dynamin- related protein 1 (DRP1) (MW 78-82 kDA) is known as the principle protein responsible for mitochondrial fission (fragmentation). This protein is mainly located in the cytoplasm and can, when activated, form ring-like multimers that translocate to the mitochondria. It is therefore mainly known for its role in *mitophagy* (Westermann, 2010). Another unique characteristic of this protein is the fact that an increase in DRP1 phosphorylation implies a decrease in DRP1 activity. Purnell *et al*. embarked on a study investigating how *autophagy* would affect the endogenous levels of DRP1 (Purnell and Fox, 2013). Bafilomycin A1 and Chloroquine (both inhibitors of autophagic degradation), as well as genetic manipulation were used and it was found that:

- The inhibition of autophagosomal-lysosomal degradation (with Bafilomycin A1 and Chloroquine) increases total DRP1 levels.
- Autophagy inhibitors as well as silencing of a gene required for autophagy initiation result in increased total DRP1 levels. Blocking autophagy therefore increases mitochondrial DRP1.
- Induction/activation of autophagy (in neurons) was able to decrease total DRP1 which corresponded with an increase in LC3B levels. This effect (in cells) was time dependent and the DRP1 response was only seen 24 hours after treatment.
- Increased levels of total DRP1 in Chloroquine-treated cells corresponded with increased LC3B levels.
- Several of the earlier studies did not evaluate DRP1 phosphorylation. While increased phosphorylation is indicative of a reduction in DRP1 activity, increased levels of the protein may indicate autophagy inhibition.

Taken together, the results of this study imply that autophagy targets DRP1 for lysosomal degradation. It also suggests that DRP1 expression is not only dependent on mitophagy but also depends on autophagy (Purnell and Fox, 2013). Interpretation of DRP1 levels in terms of autophagy, according to this study, will thus be similar to the interpretation of p62 activity during autophagy.

Table 7.5: Summary of the significant changes for p/tDRP1 ratio and total DRP1, in the different drug groups, during early and late reperfusion

Protein	Early reperfusion						Late reperfusion					
	3MA	3MA+CQ	R	R+CQ	RR	RR+CQ	3MA	3MA+CQ	R	R+CQ	RR	RR+CQ
p/t DRP1 ratio	x	x	x	↑	x	↑	↑	↑	↓	↑	x	x
Total DRP1	x	x	x	x	x	x	↓	x	x	x	x	x

Abbreviations: p/tDRP1 ratio = Phosphorylated DRP1/ total DRP1, 3MA: 3Methyl-adenine, R: Rapamycin at 1 nM, RR: Rapamycin at 250 nM for extended exposure, CQ: chloroquine, x: no change, down arrow: significant decrease in autophagic activity when compared to post ischemic/ no drug group, up arrow: significant increase in autophagic activity when compared to post ischemic/ no drug group.

An expected increase in flux (as suggested by Purnell *et al*.) is supported by the cytosolic p/tDRP1 ratio in the early reperfusion with 1 nM and 250 nM Rapamycin, as well as the late reperfusion 1 nM Rapamycin group. This increase in flux was not found in the late 250 nM Rapamycin group. The absence of increased flux in the (late reperfusion) 250 nM Rapamycin group is unexpected and should be further investigated.

The increase in p/tDRP1 ratio for steady state and flux in the late reperfusion 3MA group is also unexpected, but it confirms the tendencies observed using the LC3B and ULK1 ratio as indicators of autophagy.

Using cells and an incubation period of 24h, Purnell and colleagues evaluated *total* DRP1 levels, not the p/tDRP1 ratio, which complicates interpretation of our data. There is however no other literature available on the interpretation of DRP1 levels in autophagy, to the best of our knowledge. The *total* DRP1 levels displayed in Table 7.5 also did not correspond with the earlier mentioned findings in Purnells study (Purnell and Fox, 2013).

7.4.6. Phosphorylated/total ULK1 ratio (p/tULK1)

ULK1 (Atg1) (MW 150 kDA) fulfills an early and important role in the autophagic process. Multiple ULK isoforms exist in mammalian cells, but ULK1 and ULK2 are the only two that have a domain able to bind ATG13 and FIP200 (Chan and Tooze, 2009). ULK1 is most probably the predominant isoform involved in autophagy, as ULK2 knockdown does not affect ATG9 movement (Chan, Kir and Tooze, 2007). (Refer to Chapter 1 for more detail on the role of ULK1 in autophagy.)

Potential **problems** in tracking ULK1 activity include the fact that endogenous ULK1 is present in very low quantities, which makes the detection of phosphorylated-ULK1 (by western blot analysis) very challenging (as confirmed in this study). ULK1 is also phosphorylated by multiple kinases, and monitoring changes in phosphorylation may therefore not be informative. In addition, ULK1 has other functions in addition to autophagy and these non-autophagic functions may be reflected in its measured activity (Mochizuki *et al.*, 2011; Klionsky *et al.*, 2012).

ULK1 is known for playing a very prominent role in induction of the alternative autophagy pathway (Nishida *et al.*, 2009; Shimizu, 2018). However, the use of ULK1 activity as a tool to monitor autophagy is currently limited because the importance of ULK1-dependent phosphorylation is still unknown and only a few physiological substrates (possibly Rab9) have been identified. However, it appears as if ULK1 activity is increased with autophagic *induction*, irrespective of whether the conventional or alternative autophagic pathway is followed. In essence, it may give insight into *induction* of autophagy, but is not representative of the whole autophagic process (Klionsky *et al.*, 2012).

Table 7.6: Summary of the significant changes for p/tULK1 ratio, in the different drug groups, during early and late reperfusion

Protein	Early reperfusion						Late reperfusion					
	3MA	3MA+CQ	R	R+CQ	RR	RR+CQ	3MA	3MA+CQ	R	R+CQ	RR	RR+CQ
p/t ULK1 ratio	x	x	x	x	↓	↓	↑	x	x	x	x	x

Abbreviations: ULK1 ratio = phosphorylated ULK1/ total ULK1, 3MA: 3Methyl-adenine, R: Rapamycin at 1 nM, RR: Rapamycin at 250 nM for extended exposure, CQ: chloroquine, x: no change, down arrow: significant decrease when compared to post ischemic/ no drug group, up arrow: significant increase when compared to post ischemic/ no drug group.

As a whole, our interventional results for p/tULK1 ratio were largely disappointing. (Table 7.6) The poor quality of the phosphorylated ULK1 blots, for the reasons explained earlier, may well have affected the outcome. The significant changes in p/tULK1 ratio results include the increase in steady state in the late 3MA group, and the decrease in steady state and flux in the early reperfusion-250 nM Rapamycin group. Both these observations are supported by other “alternative pathway” proteins in the specific perfusion group and will be referred to again in the section below.

7.4.7. Collective autophagy trends as found in the separate perfusion groups

When discussing the general trends suggested by the different protein markers of autophagy, preference was given to discussing the control and the interventional groups separately.

Table 7.7: Summary of the significant changes for the different signaling proteins in the control experiments, with and without CQ, following early and late reperfusion

Protein	Early reperfusion		Late reperfusion	
	C	C + CQ	C	C +CQ
LC3 B/A ratio	x	x	↑	↑
LC3B	x	↑	↑	↑
P62	↓	x	x	x
Beclin	x	x	↑	x

Abbreviations: C: Control, CQ: chloroquine, x: no change, down arrow: significant decrease in autophagic activity when compared to post ischemic/ no drug group, up arrow: significant increase in autophagic activity when compared to post ischemic/ no drug group.

In the **control experiments,** following 20 min of global ischemia, there was a significant increasing trend in autophagy with an increase in reperfusion time: this is supported by p62, Beclin, LC3 B/A ratio and LC3 B for steady state and p62, LC3 B/A ratio and LC3 B for flux (Table 6.5, not displayed in Table 7.7). The increase in autophagic steady state at late reperfusion was supported by Beclin, LC3 B/A ratio and LC3B, the increase in flux was only maintained in the LC3 B/A ratio and LC3B groups (Table 7.7).

As discussed in Chapter 4, the response of autophagy (and flux) following ischemia and reperfusion is still controversial. It depends on the experimental model used, and the degree of ischemia and reperfusion. The different mechanisms underlying autophagic induction during ischemia (AMPK mediated) and reperfusion (Beclin mediated), should also be considered. Studies of cardiomyocytes subjected to ischemia and reperfusion have, for example, been shown to accumulate autophagosomes, (Hamacher-Brady, Brady and Gottlieb, 2006a), displayed impaired 'autophagic flux' in vitro and intact flux in vivo (Matsui *et al.*, 2007; Hariharan, Zhai and Sadoshima, 2011; Ma, Liu, Foyil, Godar, Weinheimer and Diwan, 2012).

Our findings lend support to an increase in autophagy steady state *and* flux during reperfusion, this was more prominent with an increase in reperfusion time.

Considering signaling protein activity following the administration of **3MA and CQ** the following autophagic trend(s) were the most noteworthy (Table 7.8).

- The increase in autophagy observed in control hearts during early reperfusion disappeared.
- p62 activity supports an expected decrease in flux following early and late reperfusion.
- Although there are three proteins (LC3B, p/tULK1 ratio, and p/tDRP1 ratio) supporting an increase in steady state during late reperfusion, this did not translate into an increase in flux – which is the ultimate goal.

Thus, we concluded that 3 Methyl-adenine "reversed" the increase in autophagy normally present with an increase in reperfusion time. It however did not result in a significant decrease in autophagy (steady state or flux) when compared to the no drug group.

Table 7.8: Summary of the significant changes for the different signaling proteins following 3MA administration, with and without CQ, during early and late reperfusion

Protein	Early Reperfusion		Late Reperfusion	
	3MA	3MA+CQ	3MA	3MA+CQ
LC3 B/A ratio	x	x	x	x
LC3B	x	x	↑	x
P62	x	↓	↓	↓
Beclin	x	x	x	x
p/t ULK1 ratio	x	x	↑	x
p/t DRP1 ratio	x	x	↑	↑
Rab9	↓	↓	x	x

Abbreviations: 3MA: 3Methyl-adenine, CQ: chloroquine, x: no change, down arrow: significant decrease in autophagic activity when compared to post ischemic/ no drug group, up arrow: significant increase when compared to post ischemic/ no drug group.

Another interesting finding was the significant decrease in flux and steady state in Rab9 activity during *early reperfusion only*. This strongly suggests a decrease in the alternative autophagic pathway.

Assessing the signaling proteins in combination during late reperfusion following 3MA administration, LC3B, p/tULK1 ratio, p62 and p/tDRP1 ratio support an increase in autophagic steady state. The suggested increase in steady state in late reperfusion-3MA may be attributed to the dual role of 3MA (discussed in section 7.5 (b)) during which

autophagic induction following 3MA administration (under nutrient rich conditions) is described (Wu *et al.*, 2010). As mentioned, this pronounced increase in steady state was however not translated to flux, and therefore does not represent an increase in functional autophagy.

In summary:

- In general, 3MA abolished the increase in autophagic activity that was found with an increase in reperfusion time in the control experiments.
- The administration of 3MA during *early* reperfusion may also be responsible for the inhibition of the alternative autophagic pathway, as indicated by the reduction in Rab9 expression.
- p62 activity supported an expected decrease in flux.
- During late reperfusion, although not supported by LC3 B/A ratio, four other proteins supported an increase in autophagic steady state, which did not translate in flux.

Table 7.9: Summary of the significant changes for the different signaling proteins following Rapamycin administration, with and without CQ, during early and late reperfusion

Protein	Early reperfusion				Late reperfusion			
	R	R+CQ	RR	RR+CQ	R	R+CQ	RR	RR+CQ
LC3 B/A ratio	x	x	x	x	x	x	x	↑
LC3B	x	x	x	x	x	x	x	x
P62	↑	↓	↑	↓	x	x	x	↑
Beclin	x	↑	x	x	x	x	x	x
p/t ULK1 ratio	x	x	↓	↓	x	x	x	x
p/t DRP1 ratio	x	↑	x	↑	↓	↑	x	x
Rab9	x	x	↓	↓	↑	x	x	↓

Abbreviations: R: Rapamycin at 1 nM, RR: Rapamycin at 250 nM, CQ: chloroquine, x: no change, down arrow: significant decrease in autophagic activity when compared to post ischemic/ no drug group, up arrow: significant increase in autophagic activity when compared to post ischemic/ no drug group.

Following **1 nM Rapamycin** administration in early reperfusion, p62 analysis supported an increase in steady state but a decrease in autophagic flux. This was contrary to the expected result, and contradicted by the increase in flux suggested by Beclin and p/tDRP1 ratio. In the late reperfusion 1 nM Rapamycin group, the p/tDRP1 ratio suggested a decrease in steady state and an increase in flux, while the Rab9 activity supports an increase in steady state for the alternative pathway for autophagy. These results (in both reperfusion periods), in combination with the inexplicable loss of increased autophagy with increased reperfusion time (as supported by LC3 in the control groups), prompted us to repeat the interventional experiments for autophagic induction by means of Rapamycin.

After unconvincing results obtained with **100 nM Rapamycin** (Figure 6.34 A and B), we increased the Rapamycin dosage to **250 nM**, and exposed the hearts to the drug for 30 min instead of 10 min as per the original protocol. Following early reperfusion**,** the p62 and DRP1 ratio results were similar to that found in the 1 nM Rapamycin group. The response for both these proteins are time dependent (as referred to before), and freeze clamping the hearts immediately following drug administration may render these results unreliable. The decrease in steady state and flux of the alternative autophagy pathway (according to Rab9) were however different to that of the lower Rapamycin dosage group. The decrease in steady state and flux as supported by p/tULK ratio can act as confirmation for the decrease in the conventional as well as alternative pathway in this group. Following late reperfusion**,** both LC3 ratio and p62 activity supported a predicted increase in autophagic flux.

In summary, the administration of 1 nM Rapamycin gave inconsistent and unexpected results. 250 nM Rapamycin, following early reperfusion, resulted in a decrease in the conventional and alternative autophagic pathway. Late administered 250 nM Rapamycin however resulted in an increase in autophagic flux, the desired effect. We are of the opinion that the time dependency associated with Rapamycin use (section 7.5.1) is the main contributor to the unpredictable effects during early reperfusion. This may explain why 250 nM Rapamycin had the desired effect during late, but not early, reperfusion.

Unfortunately, no studies could be found using a similar experimental model, inducing ischemia and reperfusion while manipulating autophagy with Rapamycin and 3MA during reperfusion.

7.5. POTENTIAL EFFECTS OF THE UTILIZED PHARMACOLOGICAL AGENTS

7.5.1. Rapamycin

Currently Rapamycin, an allosteric inhibitor of mTORC1 (mammalian target of Rapamycin complex 1), is the most commonly used inducer of autophagy (Klionsky *et al.*, 2016).

Rapamycin has been used in an effort to improve cardiac function. Beneficial and detrimental effects (on infarct size) have been reported following Rapamycin administration (and will be discussed in more detail in section 7.6) (Khan *et al.*, 2006; Bose *et al.*, 2007; Raphael *et al.*, 2008; Buss *et al.*, 2009; Chen *et al.*, 2013). Autophagic activity is however not measured in all the studies, and the Rapamycin effect in those studies is attributed to mechanisms unrelated to autophagy (Khan *et al.*, 2006; Bose *et al.*, 2007; Raphael *et al.*, 2008).

The inconsistency in these and other results may be attributed to several factors including the time of Rapamycin administration, the fact that the drug only causes partial inhibition of mTOR, the existence of alternative autophagic pathways that are not induced by Rapamycin, the time course of its effects, indirect drug effects and the dosage, duration of exposure and mode of administration. These factors are discussed in the following section.

1. Time of administration of Rapamycin.

Literature suggests that the most pronounced effects of Rapamycin administration will occur at the time when mTOR activity is at its highest. In terms of ischemia and reperfusion this implies extended ischemia and a long reperfusion period.

A study demonstrating the importance of timing of Rapamycin administration, to allow for maximal autophagic changes during reperfusion, was done by Wu *et al*. (2014). They showed that Rapamycin, when administered during the late phase (6–21 days) of acute myocardial infarction, attenuated cardiac dysfunction. In this in vivo study, myocardial infarction was induced in mice by permanent ligating the left anterior descending coronary artery. Drug administration and follow up occurred for 21 days after coronary ligation (Wu *et al.*, 2014).

In view of above (despite the differences in experimental models), the failure of Rapamycin to induce autophagy in our model, may be due to the fact that it was administered too early during reperfusion and could partially explain why an increase in flux was achieved during

late reperfusion only. It however cannot justify why the increase in autophagy that was seen during late reperfusion in the control experiments was lost with Rapamycin at 1nM.

2. Rapamycin causes partial inhibition of mTOR

Much of our current knowledge about mTOR comes from studies done in an effort to understand the mode of action of Rapamycin (Li, Kim and Blenis, 2014). Rapamycin binds to its intracellular receptor, FK506-binding protein 12 (FKBP12), to form the FKBP12–rapamycin complex which binds to the FKBP12–rapamycin binding (FRB) domain of mTOR (Chen *et al.*, 1995). mTOR consist of two complexes: mTOR complex one (mTORC1) and mTOR complex two (mTORC2) (Sarbassov, Ali and Sabatini, 2005).

mTORC1 is highly sensitive to Rapamycin inhibition, whereas mTORC2 is resistant to the acute Rapamycin administration. mTORC1 consists of 3 parts: mTOR, raptor (regulatory associated protein of mTOR) and GβL (G-protein β-subunit like protein). The Rapamycin-insensitive complex (mTORC2) also consists of mTOR and GβL, but rictor instead of raptor (Sarbassov, Ali and Sabatini, 2005).

The short term binding (or administration of low dose) (Yellen *et al.*, 2011) of Rapamycin causes conformational changes by (partially) disrupting the association between mTORC1 and Raptor, but not between mTORC2 and Rictor (H. Yang *et al.*, 2013). Prolonged Rapamycin exposure (24 hours) may be responsible for mTORC2, in addition to mTORC1, inhibition in some cell lines (Sarbassov *et al.*, 2006).

Studies (Sarbassov, Ali and Sabatini, 2005; Toschi *et al.*, 2009) suggest a complex mode of action for the inhibition of mTOR by Rapamycin that can vary with dosage, duration of exposure and cell type (Figure 7.2). The best studied mTORC1 substrates are S6K and 4E-BP1. Partial dissociation of mTOR and Raptor, following a low dose of Rapamycin, is enough to prevent S6K subunit, but not 4E-BP1, phosphorylation (Yellen *et al.*, 2011). Much higher Rapamycin doses may be responsible for complete mTOR- Raptor dissociation, consequently suppressing phosphorylation of both S6K and 4E-BP1 subunits. This could be as a result of a weaker interaction between Raptor and S6K than between Raptor and 4E-BP1 (Choo *et al.*, 2008).

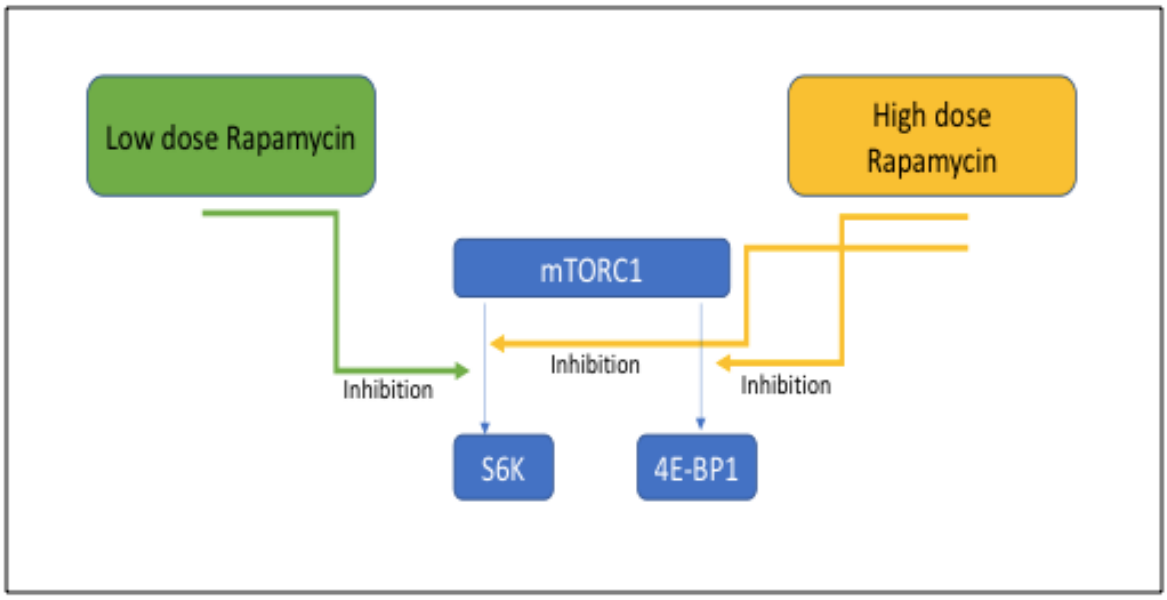


Figure 7.2: Illustration of the result of low vs high dose of Rapamycin on the mTORC1

A low dose will only inhibit S6K phosphorylation while a high dose will inhibit both S6K and 4E-BP1 phosphorylation. Abbreviations: mTORC1: mammalian target of Rapamycin complex.

Thoreen and Sabatini (2009) confirmed this by illustrating that a significant part of mTORC1 is resistant to inhibition by Rapamycin. This study was done by using a novel ATP-competitive inhibitor named Torin1 which could block the mTORC1 functions that were Rapamycin resistant (Thoreen and Sabatini, 2009).

No response following Rapamycin administration might be attributed to Rapamycin-insensitive cells in a particular cell line, which implies cells in which Rapamycin is not sufficient or effective to induce autophagy. Following an insufficient Rapamycin response, a repetition of the experiments using Torin would differentiate between mTOR-independent (Rapamycin-insensitive) or too little Rapamycin (Klionsky *et al.*, 2016).

Another potential option is to evaluate the ULK1 response since it is directly regulated by mTOR. The problems associated with ULK1 (and especially pULK1), as discussed before, will however limit its use.

3. The alternative autopahgic pathway is not activated by Rapamycin.

The Atg5/Atg7-independent (alternative) autophagy pathway is strongly activated by starvation and the stress-inducing reagent etoposide, but not by Rapamycin. An increase in Rab9 (alternative autophagic pathway activation) would therefore not be expected following Rapamycin administration (Juenemann and Reits, 2012), as was also observed in our study.

As demonstrated above, 250 nM Rapamycin (Table 7.9) had an inhibitory effect on Rab9

during early and late reperfusion. Unfortunately, as far as we are aware, there are no other studies available that investigated the effect of Rapamycin administration on Rab9 (alternative pathway) levels.

4. Time course of Rapamycin effects.

In addition to the fact that Rapamycin is a weak inducer of autophagy, its induction of autophagy by this drug is relatively slow and has been described as transient (Klionsky *et al.*, 2016).

In view of the transient action of rapamycin, evaluation of its effects in the present study may have been done too soon in early reperfusion (not allowing enough time for drug effect) and/or too long after its administration in late reperfusion (as a consequence of the transient drug effect) to enable us to capture the true drug effects (Refer to Figure 7.2). These uncertainties may have limited our ability to capture the true drug effects.

5. Indirect effects secondary to Rapamycin.

Another potential influencing factor is the indirect drug effects following Rapamycin administration. mTOR is a major regulatory protein and forms part of several signaling pathways, including those that respond to insulin, epidermal growth factor and amino acids. It is therefore involved in control processes other than autophagy and has the ability to affect many other metabolic pathways. The effect of mTOR on protein synthesis, in particular, may be a confounding factor when analysing the autophagic effects of Rapamycin (Li, Kim and Blenis, 2014; Klionsky *et al.*, 2016).

6. Dosage, duration of exposure and mode of administration.

The role players in terms of Rapamycin's dosage include

- The dosage requirement for effective suppression of mTOR varies in the different cell lines.
- Inhibition of mTOR phosphorylation of individual substrates by Rapamycin is dose-dependent.
- Sensitivity of mTORC1 and mTORC2 to Rapamycin differs (Mukhopadhyay *et al.*, 2016).

Explanation for the above are as follows: competition exists between Rapamycin and phosphatidic acid (PA) for mTOR: they share the same binding site, (Fang *et al.*, 2001) but

have opposite effects on mTOR. Rapamycin destabilizes and PA stabilizes both mTOR complexes. PA is a lipid metabolite produced by both the phospholipase D (PLD) catalyzed hydrolysis of phosphatidylcholine (PC) and during membrane phospholipid biogenesis (Fang *et al.*, 2001; Toschi *et al.*, 2009; Foster *et al.*, 2014). This competition between Rapamycin and PA implies that changes in cellular PA levels can influence the concentration of Rapamycin required to suppress mTOR (Toschi *et al.*, 2009; Mukhopadhyay *et al.*, 2016).

The higher sensitivity of mTORC1 for Rapamycin is consistent with its lower affinity for PA, which allows access to Rapamycin upon dissociation of PA. Similarly, a higher affinity of PA to mTORC2 is consistent with lower sensitivity to Rapamycin, as PA will rarely dissociate from mTORC2 to allow access to rapamycin. The key point is the opposing effects of Rapamycin and PA, and the fact that the pre-existing cellular PA levels may influence the efficacy of Rapamycin dose (i.e. the lower the PA levels, the less Rapamycin will be needed) (Foster and Toschi, 2009; Toschi *et al.*, 2009; Mukhopadhyay *et al.*, 2016).

Both the duration of Rapamycin exposure as well as its delivery mode play important roles in its end effect. These two considerations, in combination with the dose will determine the drug concentration at the effector site. The drug concentration of Rapamycin, in combination with the endogenous and opposing PA levels, will determine if partial (S6K phosphorylation only) or complete (phosphorylation of S6K and 4E-BP1) inhibition of mTORC1 will occur.

All the above confounding factors, in terms of Rapamycin's effect on autophagy, may provide an explanation for:

a) The conflicting results following Rapamycin administration in our study.
b) The variation in dosage, mode of administration and duration of drug exposure (refer to Table 7.10) reported in the literature.

Table 7.10: Illustration of the wide range of dosages, mode of administration and exposure time for Rapamycin

Rapamycin dosage	Mode of administration	Duration of exposure	Reference
0,25, 2 and 5 mg/kg	Intraperitoneal injection	30 min before heart isolation	(Yang *et al.*, 2010)
0,5 mg/kg	Intraperitoneal injection	Eight weeks	(Gu *et al.*, 2016)
0,25-0,5 mg/kg	Intraperitoneal injection	Three days	(Wang *et al.*, 2015)
0,25 mg/kg	Intraperitoneal injection	28 days	(Das *et al.*, 2014)
0,25 mg/kg	Intraperitoneal injection	30 min before heart isolation	(Khan *et al.*, 2006)
0,25 mg/kg	Intraperitoneal injection	60 min before heart stabilisation	(Das *et al.*, 2012)
1 nM	Langendorff apparatus, isolated perfused hearts	Post stabilization, pre-ischemia	(Mittal *et al.*, 2016)
0,5 nM	Isolated heart preparation (in vitro)	170 min – during the whole procedure	(Bose *et al.*, 2007)
0,5 nM	Isolated perfused rat hearts	First 15minutes of reperfusion	(Hausenloy, Mocanu and Yellon, 2004)
1 nM	Langendorff perfusion of isolated perfused rat hearts	40 min pre-ischemia	(Loos *et al.*, 2011)
1 nM	Isolated perfused rat hearts	For 30 min, 5 min ischemia and 25 min of reperfusion	(Jonassen *et al.*, 2001)
10^{-9} M	Langendorff perfusion system, rat hearts	For 30 min, 15 during ischemia and first 15 min of reperfusion	(Kleinz and Baxter, 2008)

Abbreviations: Min: minutes.

We based our original Rapamycin dosage (1 nM) on three different studies which used approximately the same experimental model (Jonassen *et al.*, 2001; Loos *et al.*, 2011; Mittal *et al.*, 2016). After obtaining the disappointing results following treatment with 1 nM Rapamycin, the aim was to obtain a proper Rapamycin response (i.e. upregulation of autophagy) without deviating from our hypothesis and our experimental model. Thus, establishment of the correct dosage, administered during *reperfusion* was of crucial importance in our quest to determine whether the manipulation of autophagy during *reperfusion* is cardioprotective. This prevented us from administering Rapamycin intraperitoneally, as was done in a number of other studies (Table 7.10). We therefore increase the dose to 100 nM. Again, the results were discouraging. That prompted a further increase in the dose (to 250 nM), in addition to an extended exposure time (from 10 min to

30 min). We would have preferred to only change one variable at a time, but practical and financial implications rendered it impossible. Administration of 250 nM Rapamycin for an extended exposure did result in autophagic flux induction during late reperfusion.

7.5.2. 3 Methyl-adenine

The fact that 3MA is the most widely employed autophagy inhibitor, prompted us to use it in the present study.

The most significant findings using 3MA as an autophagy inhibitor include:

- During early reperfusion; a disappearance of the increase in autophagy with an increase in reperfusion time as demonstrated in the control experiments.
- A decrease in Rab9 levels, during early reperfusion, suggestive of a reduction in the alternative pathway.

3MA administration therefore mostly met the expected outcomes during early reperfusion.

3MA was first discovered in 1982 and is still the most widely used autophagic inhibitor (Seglen and Gordon, 1982). Three classes of phosphoinositide 3-kinase (PI3K) exist: Class I PI3K is an autophagic inhibitor, (Martelli *et al.*, 2012) class II PI3K activity is of no significance to autophagic control, and class III PI3K is an activator of autophagy required for early autophagosome formation (Miller, Tavshanjian, *et al.*, 2010). 3MA, as well as Wortmannin and LY294002, are class III PI3K inhibitors, and therefore responsible for the suppression of autophagy (Y. Yang *et al.*, 2013).

Current strategies to inhibit autophagy include class III PI3K-inhibitors, lysosomal enzyme inhibitors and genetic approaches (Vinod *et al.*, 2014). When using 3MA the following is important to bear in mind:

1. 3MA's effect on class I PI3K (Figure 7.3)

Most of the information about the role of autophagy was obtained from its inhibition through blocking class III PI3K. 3MA is however not a specific autophagy inhibitor, and is also known to interfere with the activity of class I PI3K (Lindmo and Stenmark, 2006). The potential problem is that class I PI3K inhibition is able to reduce the activity of its downstream target protein kinase B (PKB). PKB plays an important role in signal transduction, activating a wide range of proteins involved in growth and survival (Song, Ouyang and Bao, 2005). Using cell lines dependent upon PKB signaling as experimental model may therefore respond to 3MA

in several ways, apart from inhibition of autophagy (Vinod *et al.*, 2014).

Klionsky *et al*. also noted 3MA's inhibitory effect on class I PI3K. They mentioned that this may result in autophagic induction when given chronically at a low concentration (Klionsky *et al.*, 2012). (This is however not relevant in our study.)

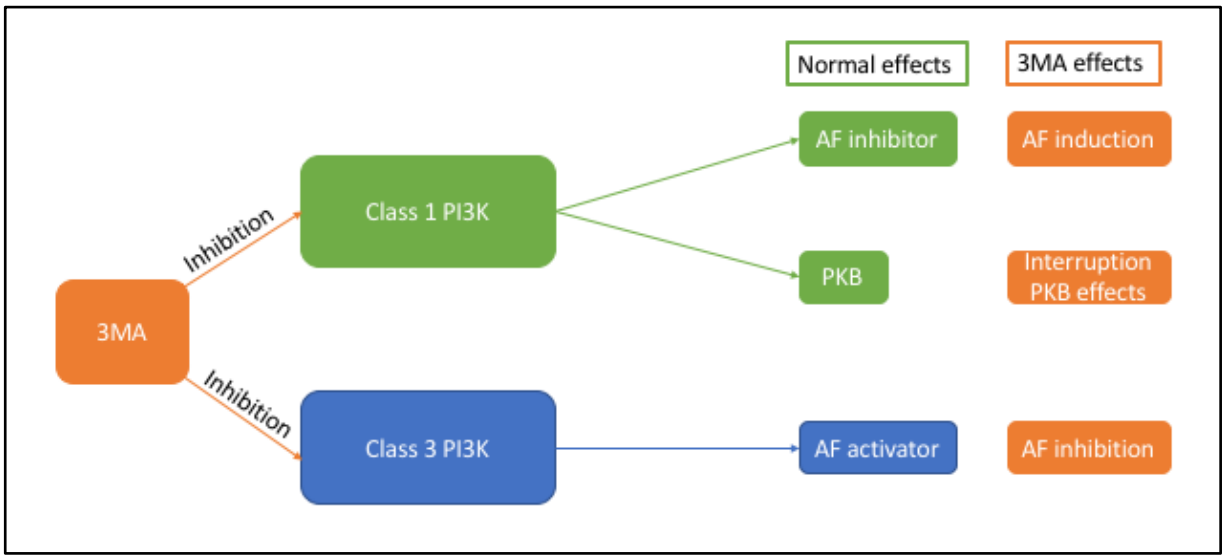


Figure 7.3: Graphic representation of the inhibitory effects of 3MA on both class 1 and 3 PI3K

The potential consequences include induction of autophagy, inhibition of autophagy and the interruption of PKB effects. Abbreviations: 3MA: 3 Methyl-adenine, PI3K: phosphoinositide 3-kinase, AF: autophagy, PKB: protein kinase B.

2. Limitations around using a class III PI3K-inhibitor

As stated above, class III PI3K has other functions apart from autophagic regulation. In addition to its role in autophagy, is it also responsible for the regulation of cellular membrane trafficking processes (Johnson *et al.*, 2006). In non-conventional autophagy, class III PI3K is not even required for autopahgic activation, therefore 3MA cannot guarantee complete autophagic blockage (Francesca Scarlatti *et al.*, 2008). Of the alternative autophagic pathways, the Atg5/Atg7-independent pathway is 3MA sensitive, while the Beclin 1-independent pathway is 3MA insensitive but still depends on LC3 (Dupont and Codogno, 2013). Thus, awareness of the type of autophagy induced is required before choosing the inhibition route.

All of the above contribute to the view point that class III PI3K-inhibitor is a "messy" drug when aiming at autophagic inhibition. Nevertheless, class III PI3K-inhibition is still the most widely used method of autophagic inhibition and most of our current knowledge re autophagy is derived from studies aimed at manipulation of this target (Vinod *et al.*, 2014).

3. 3MA's role in apoptosis

3MA itself can induce cell death and apoptosis directly, irrespective of its relationship with autophagy (Sheng *et al.*, 2013). Autophagy and apoptosis however also have an inverse relationship (up to a point), which may explain why the pharmacological induction of apoptosis in cells is enhanced by 3MA (an inhibitor of autophagy). A reduction in autophagy may contribute to an increase in apoptosis, (Liu *et al.*, 2011) a finding also supported by another study (Li *et al.*, 2009).

3MA may therefore induce apoptosis in both an indirect and direct manner (Davi *et al.*, 2008; Sheng *et al.*, 2013; Vinod *et al.*, 2014).

Other studies however support the opposite by stating that 3MA arrests autophagy and *prevents* apoptosis by inhibiting cathepsin B and cytochrome *c* release (Canu *et al.*, 2005; Kunchithapautham and Rohrer, 2007; Wang *et al.*, 2011).

4. The dual role of 3MA in autophagy

Although all of the tested PI3K inhibitors (3MA, Wortmannin and LY294002) target both class I and class III PI3K indiscriminately (Knight and Shokat, 2007; Kong and Yamori, 2008), the suppression of autophagy is dependent on class III PI3K inhibition.

Wu *et al*. (2010) discovered that 3MA may however *induce* autophagic flux when the experimental cells underwent prolonged (3MA) treatment in a nutrient rich medium. The normally predicted outcome (autophagic inhibition) following 3MA administration occurred only in the starvation-induced autophagy group. It appears that under such specific treatment conditions, 3MA acts in a manner similar to Rapamycin. Wortmannin, however, suppressed autophagy regardless of the nutrient status (Wu *et al.*, 2010).

The above is attributed to 3MA's differential time-based effects on class I and class III PI3K: 3MA persistently suppresses class I PI3K, but only transiently suppresses class III PI3K. The inhibitory effects of Wortmannin were found to those of 3MA: persistent on class III, and transient on class I PI3K. These findings are supported by earlier studies (Powis *et al.*, 1994; Petiot *et al.*, 2000; Tassa *et al.*, 2003). Based on their results Wu *et al*. (2010) advised that Wortmannin may be the more suitable autophagy inhibitor due to its more persistent inhibition of class III PI3K (Figure 7.4). The latter study was however done in cells with a 3MA exposure time of 9 hours. How to translate this to our experimental model is difficult to predict. To further evaluate the dual role of 3MA, in our study, all 3MA experiments would have to be repeated with Wortmannin. The significance of this dual role of 3MA may be

relevant in our study since our rats had free access to food and water (Wu *et al.*, 2010).

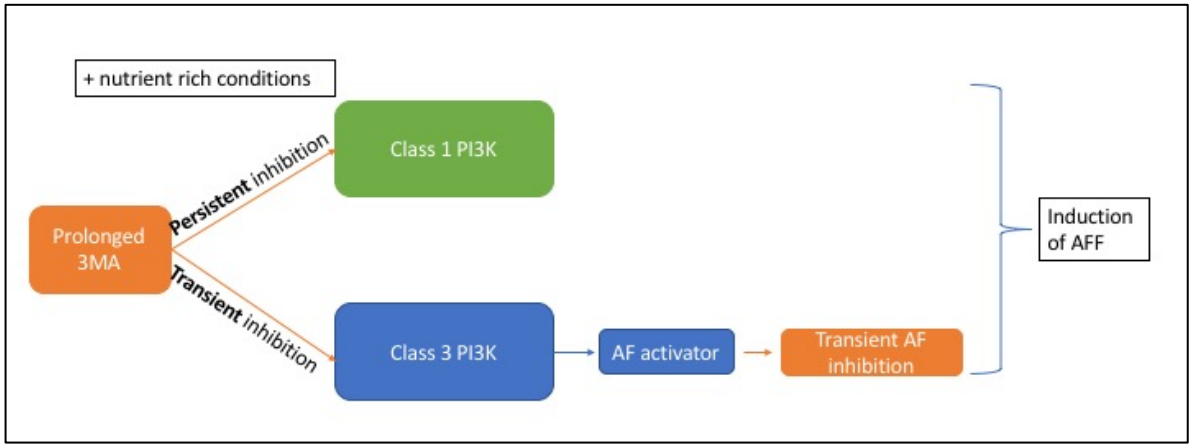


Figure 7.4: Illustration of the dual role of 3MA when administered over a long period in nutrient rich conditions

This is attributed to the transient inhibition on class III PI3K. Abbreviations: 3MA: 3 Methyl-adenine, PI3K: phosphoinositide 3-kinase, AF: autophagy, AFF: autophagic flux.

Table 7.11 serves as an illustration of the wide range of doses, different methods of administration as well as exposure time in terms of 3MA administration to induce inhibition of autophagy. In the experimental models that correlated best with ours the doses ranged from 1-10 mM. We used 3MA at a concentration of 2.5 mM and would have not been able to increase the dose because of financial constraints. Intra-peritoneal as well as chronic (daily) administration were also not an option since our study focus was on interventions *during reperfusion*.

Considering all of above it is understandable that finding an appropriate and specific method to inhibit autophagy is referred to as “finding a fitting shoe for Cinderella” (Miller, Oleksy *et al.*, 2010).

Table 7.11: Illustration of the wide range of dosages, method of administration and time of exposure for 3 Methyl-adenine

Dosage	Method of administration	Duration of exposure	Reference
10 mg/kg	Intra-peritoneal injection	pre-experimentation	(Lekli *et al.*, 2009)
10 mM	Cardiomyocytes		(Valentim *et al.*, 2006)
10 mM	Langendorff perfusion	10 min pre-ischemia	(Yang *et al.*, 2010)
1 mM	Langendorff perfusion	40 min pre-ischemia	(Loos *et al.*, 2011)
5 mM	Cells	20 min incubation	(Heckmann *et al.*, 2013)
15 mg/kg/day	Intra-peritoneal injection	3 weeks	(Maeda *et al.*, 2013)
15 mg/kg	Intra-peritoneal injection	30 min pre-ischemia	(Wei *et al.*, 2013)
5 mM	Isolated perfused rat atria	75 min ischemia and 75 min reperfusion	(Hermann *et al.*, 2014)
4.5 mM	Intra-peritoneal injection	Pre-ischemia	(Dmitriev *et al.*, 2015)
10 mg/kg	Intra-peritoneal injection	Injected twice at 30 min and 10 min before experiment	(Lekli *et al.*, 2010)
1 mM	Langendorff perfusion	For 30 min	(Xiao *et al.*, 2012)
10 mM	Langendorff perfusion	For 10 min before ischemia	(Yang *et al.*, 2010)

Abbreviations: Min: minutes.

7.5.3. Chloroquine

CQ was employed in our study for the measurement of autophagic flux. Autophagic flux refers to the entire process of autophagy – this includes the delivery of cargo to lysosomes, via autophagosome lysosome fusion, as well as the breakdown and release of the end-products into the cytosol. The difference in autophagy activity (autophagosome abundance), in the presence and absence of CQ, was used to determine if an increase in autophagosomes resulted from:

a) An increase in production (and the whole autophagic process), or
b) Only a decrease in autophagosome breakdown (Klionsky *et al.*, 2016).

Since 'autophagy' is a process, the reliance on (only steady state) autophagosome levels may not result in reliable conclusions regarding autophagic activity (Ma, Liu, Foyil, Godar, Weinheimer and Diwan, 2012).

CQ is responsible for blocking autophagosome-lysosome fusion (Poole and Ohkuma, 1981; Kawai *et al.*, 2007). This is done by the accumulation of CQ inside the acidic lysosomes followed by raising (neutralizing) the lysosomal pH (Seglen, Grinde and Solheim, 1979;

Vinod *et al.*, 2014). It therefore acts as a late stage inhibitor of autophagy, preventing the degradation of the autophagosomes by lysosomes (Iwai-Kanai *et al.*, 2008). A potential drawback is associated with CQ's mechanism of action is that it may be able to block all (or other) acidic organelles with unpredictable and unwanted effects (Vinod *et al.*, 2014).

Other available pharmacological options for inhibiting autophagy through lysosomal blockage include Bafilomycin A1, ammonium chloride, Monensin, E64d and Pepstatin (Vinod *et al.*, 2014).

CQ is a frequently used pharmacological agent. It is used:

- To distinguish between steady state and flux.
- As a (late) autophagic inhibitor.
- In cancer therapy, (Cheong *et al*., 2012), malaria (Homewood *et al.*, 1972), rheumatoid arthritis (Kremer, 2001) and lupus erythematosus (Rainsford *et al.*, 2015; Pasquier, 2016).

Long term use of CQ has, however, been shown to have toxic effects, particularly on the eyes and heart (Fragasso *et al.*, 2009; Michaelides *et al.*, 2011). The literature available on CQ's effect on the heart is conflicting and both cardioprotective as well as cardiotoxic effects have been reported.

Cardiotoxicity to CQ may be secondary to impairment of mitochondrial antioxidant buffering capacity, an increase in oxidative stress and mitochondrial dysfunction. All of these were demonstrated in a pressure overload hypertrophy rat experimental model (Chaanine *et al.*, 2015). Cardiomyocyte hypertrophy (following 4 week oral administration) (Izunya *et al.*, 2011), cardiomyopathy (Soong *et al.*, 2007) and the induction of myocardial necrosis following the combination of CQ and intermitted hypoxia, (Maeda *et al.*, 2013) can all potentially contribute to cardiotoxicity following its administration.

Reports supporting the cardioprotective effects of the drug, include CQ being responsible for an improvement of left ventricular diastolic dysfunction, (G.-S. Zhang *et al.*, 2016) as well as the prevention of the development of pulmonary artery hypertension, right ventricular hypertrophy, and vascular remodeling after monocrotaline administration (Long *et al.*, 2013). The mechanism of action for the last mentioned protective effect includes inhibition of autophagy and lysosomal degradation of bone morphogenetic protein type II receptor (BMPR-II) (Long *et al.*, 2013). In a study done by Fazekas and Szekeres (1988), CQ played

a protective post-ischemic role in dogs and rats, which was partly explained by CQ's anti-phospholipase activity (Fazekas and Szekeres, 1988).

CQ however had no statistically significant effect on either global or regional cardiac function in our study. The reason for the discrepancy in results, between ours and above-mentioned studies, could be ascribed to the fact that different experimental models were used, CQ was administered via different routes (intra-peritoneal injection and oral route), at different doses and (especially) for longer periods of exposure.

That raises the question, did we give enough (and correctly administered) CQ to be able to induce autophagic flux? Intraperitoneally administered CQ, at 10 mg/kg, 1 hour prior to sacrificing the rat (or mouse), is well supported in the literature as a valid method to allow measurement of autophagic flux (Ma, Liu, Foyil, Godar, Weinheimer, Hill *et al.*, 2012; Zhang *et al.*, 2014; Gottlieb *et al.*, 2015).

Studies give contradicting results regarding up- or downregulation of autophagic flux, as well as the beneficial or detrimental effects thereof, during reperfusion (Matsui *et al.*, 2007; Ma *et al.*, 2015; Orogo and Gustafsson, 2015).

In our study, the most pronounced and consistent results, in terms of autophagic flux, were noted in the late reperfusion control and 250 nM Rapamycin group (table 7.12).

The results pertaining to flux further highlight that 1 nM Rapamycin (in comparison to 250 nM Rapamycin) was insufficient to induce the true autophagic process. The extent and the severity of the ischemic episode, reperfusion duration and the degree to which pharmacological manipulation will influence autophagy – will all play a role in the effect on flux.

Table 7.12: Table summarising the autophagic flux results as per the activity of the different proteins used during western blotting

	Early reperfusion				Late reperfusion			
Group	Control	3MA	R	RR	Control	3MA	R	RR
AF Flux	**+**	**-**	**++/-**	**+/--**	**++**	**+/-**	**+**	**++**

+/- refers to inconsistent or no effect, - to a decrease in autophagic flux + to an increase in autophagic flux. The amount of "-" or "+" correlates with the amount of proteins supporting the increase or decrease in flux. Abbreviations: AF Flux: autophagic flux, 3MA: 3 Methyl-adenine, R: 1 nM Rapamycin, RR: 250 nM Rapamycin.

Few reports are available on the use of Chloroquine to determine the difference between

autophagy steady state and flux in an experimental model similar to ours.

We based our Chloroquine administration on the following two studies: In 2012 Ma *et al.* investigated how impaired autophagosome clearance will contribute to cardiomyocyte death in ischemic and reperfusion injury. The mice in this experiment were subjected to reversible LAD artery coronary ligation one hour after the administration of 10 mg/kg Chloroquine (Ma, Liu, Foyil, Godar, Weinheimer and Diwan, 2012). Although they did not use the working heart model they made use of an in vivo mice model, subjected to ischemia and reperfusion, aiming to determine cumulative autophagic flux by counting the numbers of autophagosomes in the presence and absence of chloroquine. In another in vivo rat model, investigating the effect of autophagic flux on cardioprotection, 10 mg/kg CQ was injected, again one hour prior to LAD occlusion. Ischemia lasted for 30 min and reperfusion for 120 min, very similar to the durations we used (Zhang *et al.*, 2014).

Both studies display the following resemblances to our study: CQ was specifically administrated for autophagic flux purposes, in an in vivo rodent animal experimental model with induction of ischemia followed by reperfusion. This motivated us to use CQ in the same manner in which they did.

Four other studies however propose a longer period between CQ administration and measurement of autophagic parameters. Iwai-Kanai *et al*. (2008), although also using 10mg/kg CQ, waited four 4 hours before fluorescence microscopy was done on the harvested cardiac mouse tissue (Iwai-Kanai *et al.*, 2008). The aim in a study done by Kurdi and colleagues was to evaluate the effects of short-term and long-term everolimus administration on mTORC1 inhibition and autophagy induction in mice. They investigated autophagic markers in the *liver*, and there was no emphasis on ischemic reperfusion or specific measurement of autophagic flux. In this study 100mg/kg CQ was injected three hours prior to tissue harvest (Kurdi *et al.*, 2016). In an investigation done into the effect of exercise on autophagic flux, with an experimental model significantly different from ours, 50mg/kg CQ was IP administered four hours before tissue collection (Campos *et al.*, 2017). Lastly, a review article, written almost ten years ago, recommends that a minimum of two hours need to elapse between tissue harvesting and IP CQ administration for flux purposes (Perry *et al.*, 2009).

Although our CQ regime is based on two similar studies, the results following CQ administration may have to be interpreted with care. Dose response as well as different

time lapse studies are required to determine the desired CQ regime for autophagy flux purposes in the working rat heart model.

In conclusion, manipulation of autophagy with pharmacological inhibitors and activators is unfortunately associated with a number of potential confounding factors, the most important of which are:

- The impact of these drugs on other cellular pathways.
- Pleiotropic effects.
- The ideal timing of drug administration in relation to the experimental model, the hypothesis and the desired effect.
- The most desirable dose, method of administration and duration of exposure of the drug to the model.

In view of the above, it is recommended to do different dose/response, administration route and exposure duration studies. It is also advisable to use more than one method to induce or inhibit autophagy. In doing so the results can be compared effectively and it would be easier to attribute observed results to pleiotropic drug effects or to effects secondary to the manipulation of autophagy. A confirmation of the above with genetic manipulation studies may be viewed as the gold standard.

The implications of the above approach, from a time, financial and practical point of view, are self-evident.

7.6. LEFT VENTRICULAR INFARCT SIZE ANALYSIS

For analysis of the autophagic manipulation effect on the heart, following ischemia/reperfusion, use was made of measurement of infarct size. This is regarded by many to be the “gold standard” for evaluation of cardioprotection. When analysing the infarct sizes as determined in this study, the most significant finding was the reduction in infarct size observed in the early reperfusion 3MA group (Figures 6.36 & 37 and Table 6.12). The infarct size in this group was significantly smaller than the control, early and late reperfusion 1 nM Rapamycin and the late reperfusion 3MA group. Also of note was the fact that there were no significant differences in the areas at risk (Figure 6.34) between the different groups.

3 Methyl-adenine’s effect on infarct size

When evaluating the expression of the different proteins in the early reperfusion 3MA group,

the most significant findings was the reduction in the conventional and alternative (Rab9) pathway (Table 6.11). There is no literature available on the consequences following the manipulation of the alternative autophagic pathway to the best of my knowledge.

Interestingly, similar Rab9 changes were also found in the early reperfusion 250 nM Rapamycin group, where no significant reduction in infarct size was observed (Figure 6.39). I therefore conclude that the decrease in steady state and flux of the alternative pathway was probably not responsible for the reduction in the infarct size in the early reperfusion - 3MA group.

Excluding the changes in the alternative pathway, the other noticeable result in the early reperfusion 3MA group was the reversal of the increased autophagic trend associated with prolongation of reperfusion time as seen in the control group. It is therefore possible that a reduction in autophagy during early reperfusion is indeed cardioprotective. However, this change was also seen in the 1 nM and the 250 nM early Rapamycin groups where the infarct sizes did not differ significantly from the control group. The decrease in autophagy during early reperfusion is therefore unlikely to be the basis for the reduction in infarct size in the 3MA group.

If the infarct size reduction cannot be attributed to the alternative autophagic pathway or the inhibition of autophagy, drug effects may be the best explanation for the observation (refer to section 7.5 (b)). The question however is, if the protective effect is secondary to 3MA's pleiotropic drug effects, why do we not see a significant infarct size reduction in the late reperfusion 3MA group? A possible explanation for the loss in cardioprotection could be the, as yet unexplained, increase in steady state autophagy seen in the late reperfusion 3MA group.

Another possible explanation for the effects of 3MA could be its ability to induce or inhibit apoptosis, independent of autophagy (refer to section 7.5 (b)).

Previous studies aimed at assessing the cardioprotective effect of 3MA give contradicting results. Pre-ischemic administered 3MA (at 1 mM) to rat hearts undergoing 35 min of regional ischemia followed by 60 reperfusion, had no effect on infarct size (Mzezewa, Lochner and Huisamen, 2017). In an in vivo ischemic mouse model, 3MA was administered after left anterior descending (LAD) coronary artery ligation. The results showed that 3MA exacerbated post infarction cardiac remodeling and dysfunction (Wu *et al.*, 2014). In another in vivo study, 30 min of LAD ligation was followed by 120 min of reperfusion. 3MA was

administered 30 min prior to LAD ligation at a dose of 1 mg/kg. The decrease in autophagy in this study attenuated the decrease in infarct size secondary to Valsartan preconditioning which induced autophagy (Wu *et al.*, 2013).

The study models above however differ significantly from the model used in the present study. To the best of my knowledge no other studies exist assessing the effect of post ischemic administered 3MA on infarct size in a model similar to ours.

Rapamycin's effect on infarct size

None of the Rapamycin groups (1 nM and 250 nM Rapamycin in early and late reperfusion, with and without CQ) demonstrated a significant change in infarct size when compared to the control group (Figures 6.36&39 and Table 6.12&13).

Failure to demonstrate Rapamycin-induced reduction in infarct size was also reported by others. For example, in a study done by Hausenloy *et al*. (2004) isolated perfused rat hearts were used to investigate the cross-talk between kinases during early reperfusion and their contribution to preconditioning. In a subgroup of this study, hearts were subjected to 35 min of regional ischemia, infarct size measured after 120 min of reperfusion and Rapamycin (0.5 nM) was administration for the first 15 min of the reperfusion, a protocol which correlates very well with our early reperfusion 1 nM Rapamycin group. Hausenloy also found that the administration of Rapamycin did not influence infarct size when compared to the control group (Hausenloy, Mocanu and Yellon, 2004).

In contrast to the above, there are substantial support for Rapamycin-induced cardioprotection. Khan and colleagues pretreated adult mice with intraperitoneal Rapamycin (0.25 mg/kg) before being subjected to 20 min of global ischemia and 30 min of reperfusion in the Langendorff mode. The infarct size in the Rapamycin group was significantly smaller ($P < 0.001$) than in the control group. A mitochondrial K_{ATP} channel blocker however attenuated the protective Rapamycin effect (as illustrated by the increase in infarct size from 10.10 ± 2.80% to 32.20 ± 1.80%, without and with K_{ATP} channel blocker respectively, $p < 0.001$). Rapamycin's cardioprotective effect (in the above study) was therefore attributed to its preconditioning-like effect through the opening of mitochondrial K_{ATP} channels (Khan *et al.*, 2006). Rapamycin's effect on autophagy was not evaluated in this study.

Infarct sizes in rats pretreated with three different doses of intraperitoneal Rapamycin (0.25, 2, and 5 mg/kg), also demonstrated that the drug mediates cardioprotection when compared to the control group. Rapamycin was administered 30 min before heart isolation, followed

by 40 min of global ischemia and 120 min of reperfusion. In this study, the Rapamycin mediated protection was attributed to its involvement in the PI3K pathway as well as the activation of mitochondrial K_{ATP}-channels. The protection was however independent of Rapamycin-induced autophagy (Yang *et al.*, 2010).

The most significant difference between the studies above is the timing of Rapamycin administration in relation to ischemia and reperfusion. Following the same protocol as ours (administration of Rapamycin during reperfusion) yielded the same results i.e. no significant difference in infarct size associated with Rapamycin administration. Administration of Rapamycin *before* ischemia however had cardioprotective properties – most probably as a result of its preconditioning effects. These preconditioning-like cardioprotective effects of Rapamycin, against ischemia and reperfusion injury, have already been described in 2006 (Khan *et al.*, 2006).

In an *in vivo* study mice were subjected to 30 min of regional ischemia followed by 24hours of reperfusion. Intracardiac Rapamycin (0.25 mg/kg) was injected five min prior to reperfusion. This approach was adopted to ensure its immediate availability at the onset of reperfusion as well as avoiding any preconditioning effect. Rapamycin administration resulted in a significant reduction in myocardial infarct size as well as a reduction in apoptosis when compared to the control group. The Rapamycin mediated protective effect was associated with the AKT and ERK signaling pathway, suggesting that reperfusion therapy with Rapamycin protects the heart against I/R injury by selective activation of mTORC2 and ERK with concurrent inhibition of mTORC1 and p38 (Filippone *et al.*, 2017). This study, although different from ours, demonstrated that the Rapamycin administered during reperfusion (and therefore avoiding the preconditioning effect) may still have autophagy-mediated cardioprotective properties. The most important difference between this and our study might be the reperfusion period – in this study the effect of Rapamycin was assessed after 24 hours of reperfusion.

Chen and colleagues demonstrated a 45% reduction in infarct size associated with post-ischemic administration of Rapamycin in an in vivo mice model. All subjects underwent 35 min of left coronary artery ligation followed by 4 hours of reperfusion. Autophagy was measured, in vivo, with a fluorescence molecular tomography (FMT) and a cathepsin activatable fluorochrome. Their results suggest that Rapamycin induced autophagy exerts a cardioprotective effect during reperfusion injury (Chen *et al.*, 2013). This is supported by another in vivo study during which they showed that the cardioprotective effects of mTORC-

inhibitors following chronic ischemia are present up to three days after induction of ischemia. As in our study, Rapamycin was initiated post myocardial infarction in an attempt to closely resemble the clinical situation (Buss *et al.*, 2009; French, Taatjes and Sobel, 2010).

Wu and coworkers used an in vivo model to demonstrate the myocardial protective effects of chronic Rapamycin administration following acute myocardial infarction. Although they did not determine infarct sizes as part of their results, data showed that Rapamycin attenuated post-infarction cardiac remodeling and dysfunction. The cardioprotective effect was partly attributed to a decrease in the inflammatory response (NFκB activation) in the acute stage of MI following Rapamycin administration (Wu *et al.*, 2014).

In conclusion, Rapamycin administration may result in cardioprotection as a result of autophagic induction, preconditioning effects and its anti-inflammatory action. Pre-ischemic administered Rapamycin demonstrated cardioprotection mainly as a result of preconditioning. The cardioprotective effect secondary to post ischemic administered Rapamycin in the in vivo studies (which all could allow for a much longer reperfusion duration) could all be linked to an increase in autophagy. Demonstration of the cardioprotective effects (by, for example, infarct size) will depend on:

- The amount of (baseline) autophagy induced or inhibited by ischemia and/or reperfusion.
- The experimental model.
- The dose, the method, duration and timing of administration of Rapamycin and
- Timing of the response to Rapamycin.

Chloroquine's effect on infarct size

For accurate evaluation of the effects of manipulation of autophagy on infarct size, it was necessary to establish whether the experimental intervention with CQ had an effect on infarct size. This would allow for distinction between the effects of changes in steady state versus autophagic flux (i.e. changes in autophagosomes only vs functional autophagy) on infarct size. Also, without any significant change in flux, a significant change in infarct size as a result of CQ administration can contribute to the controversy regarding the protective versus detrimental effects of CQ.

The group with the most consistent and convincing increase in autophagic flux was the late reperfusion 250 nM Rapamycin group. No significant differences were found in the infarct sizes for 250 nM Rapamycin, with and without CQ, when compared to the control group.

(Figure 6.39)

None of the other CQ groups demonstrated significant changes in infarct size. (Figure 6.36) The decrease in flux found in the early reperfusion 3MA, late reperfusion 3MA and early reperfusion 250 nM Rapamycin groups, also did not demonstrate any significant changes in terms of infarct size when compared to the control group.

In conclusion, no cardioprotective or cardio-detrimental effects could be demonstrated following CQ administration, neither as a result of CQ specific drug related effects nor as a consequence of the manipulation of autophagic flux.

7.7. OTHER INFLUENCING FACTORS

7.7.1. Apoptosis

The relationship between autophagy and apoptosis or other forms of cell death is an expanding area of research. It is complex and still poorly understood. Dead and dying cells can concurrently show characteristics of autophagy, apoptosis, and necrosis. Understanding regulation of cell death by autophagy is important as we try to either prevent cells from dying (as in neurodegenerative diseases) or we want to induce cell death (as in cancer treatment) (Gump and Thorburn, 2011).

Under normal or mild stressed conditions, autophagy is responsible for the degradation and the recycling of old or damaged proteins and organelles. The balance between all the modalities responsible for cell death (autophagy, apoptosis, necrosis, necroptosis and autophagy cell death (ACD) or autosis, as discussed in Chapter 4, section 4.2) maintain cellular homeostasis. Inactivation or inhibition of autophagy may therefore be responsible for the accumulation of abnormal proteins and organelles, thereby promoting apoptosis or necrosis. Extreme autophagic activity, on the other hand, can destroy cellular organelles causing cellular dysfunction, leading to abnormal apoptosis and necrosis (Nishida, Yamaguchi and Otsu, 2008). The disruption of the autophagic/ apoptotic relationship may therefore have significant pathophysiological consequences.

This relationship is important in interpreting the results of our study. How, and to what extent, would autophagic manipulation affect apoptosis? Also, will the direct and indirect manipulation of apoptosis be able to influence the global and regional myocardial function?

Simplistically, autophagy blocks the induction of apoptosis, and activation of apoptosis can shut off the autophagic process. Autophagy may under certain circumstances however also

aid in the induction of apoptosis or necrosis, being responsible for autophagic cell death. The relationship between all of the above will influence the clearance of dying and damaged cells (Gump and Thorburn, 2011; Mariño *et al.*, 2014).

Autophagy and apoptosis often occur within the same cell, with autophagy usually preceding apoptosis (M. Maiuri *et al.*, 2007). This is because stress often stimulates an autophagic response, especially if the level of stress is not lethal. Apoptosis is activated when stress exceeds a critical duration or an intensity threshold (Mariño *et al.*, 2014).

The process of mitophagy is one of the main mechanisms in which autophagy reduces apoptotic activity. Damaged mitochondria have the potential for apoptotic activation and their autophagic removal can therefore increase the apoptotic induction threshold (Youle and Narendra, 2011; Mariño *et al.*, 2014).

Autophagy can also favour the induction of apoptosis, although usually it is responsible for the reduction in apoptotic activity. Autophagosome formation has been implicated in the activation of caspase 8, which forms a complex with the death receptor adaptor protein FAS-associated death domain (FADD) and ATG5 (Nezis *et al.*, 2010). Inhibition of the early steps of autophagy (by knockout of *Atg3* or *Atg5*) reduces the activation of caspases 8 and 3, whereas inhibition of the late steps of autophagy (by Bafilomycin A1) increased caspase-dependent cell death (Young *et al.*, 2012). Autophagy may also stimulate apoptosis by depleting endogenous inhibitors of this cell death pathway (Nezis *et al.*, 2010).

ATG proteins can influence apoptosis *independent* of the autophagic process. ATG12 activates caspases through the mitochondrial pathway and ATG7 (but not ATG5) also induces apoptosis by triggering an increase in lysosomal membrane permeability (Boya *et al.*, 2003; Kessel, Price and Reiners John J, 2012).

Following severe cellular stress, the adaptive limit from an autophagic point of view may become overwhelmed, causing suicidal cell death program (apoptosis) activation. Apoptosis is associated with a high degree of caspase activation, resulting in the cleavage of multiple proteins. Caspases can also digest the autophagic proteins, ATG3 (Oral *et al.*, 2012) and Beclin1 (Wirawan *et al.*, 2010), which may result in autophagic inactivation.

Specific scenarios of cell death may therefore involve both processes. FADD death domain can induce both apoptosis and autophagy (Thorburn *et al.*, 2005) and activation of mitochondrial c-Jun N-terminal kinase (JNK), known for playing a central role in initiating apoptosis, can induce autophagy and apoptosis (J. Xu *et al.*, 2015).

According to this, manipulation of one type of cell death mechanism would also affect the others because everything is connected. These ideas could have practical implications when we try to manipulate these pathways to better treat disease. Induction of autophagy may reduce apoptosis sufficiently to keep the cell under the threshold of caspase activity needed to cause death (Thorburn, 2008).

In addition to above, it should be kept in mind that stimulation of cell death by apoptosis and/or autophagy can also trigger necrosis (Yu *et al.*, 2006).

The questions resulting from above are:

- Will CQ administration (a late autophagy inhibitor) also increase apoptosis?
- To what extent did the manipulation of autophagy, in our study, influence apoptotic activity?
- Did the (potential) change in apoptotic activity influence the measured global and regional myocardial function?

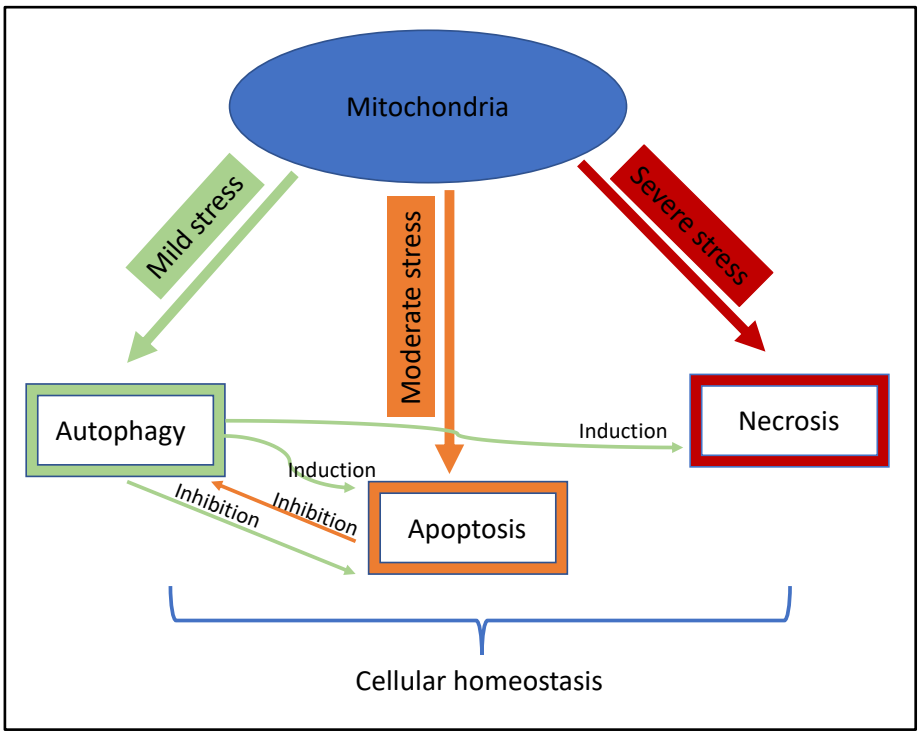


Figure 7.5: Illustration of the relationship between autophagy, apoptosis and necrosis

Autophagy is normally induced following mild cellular stress. With increasing stress apoptosis begins to occur because of cytochrome *c* release from mitochondria. Under extreme stress necrosis occurs as a consequence of ATP depletion. Normally autophagy and apoptosis are mutually inhibitory, autophagic cell death may however induce/ contribute to apoptotic (and necrotic) cell death following extreme stress conditions (Nishida, Yamaguchi and Otsu, 2008).

The answers will depend on the amount of stress in the experimental model (since this will

determine whether autophagy, apoptosis or necrosis will be induced) as well as the extent of the pharmacological manipulation of autophagy. (Figure 7.5)

Awareness of the discussed interactions are important, especially from a clinical point of view. Ideally, apoptotic markers (in conjunction with autophagic markers) have to be measured, in the control as well as the experimental groups. This will enable insight into the contribution as well as cross talk between these two processes.

In conclusion, in the majority of situations, it seems that apoptosis and autophagy are mutually inhibitory. The exception is during autophagic cell death, during which autophagy may favour apoptotic activation. Cellular stress at early time points or low doses of stress will induce autophagy while later induced, high doses of stress will induce apoptosis (Mariño *et al.*, 2014).

7.7.2. Nutritional status, physiological and physical stress.

The ability of cells to respond to changes in nutrient availability and stressors is important for the maintenance of metabolic homeostasis and viability.

One of the key cellular responses to nutrient withdrawal (starvation) is the upregulation of autophagy. In fact, starvation is the most effective known physiological inducer of autophagy. mTORC1 and AMPK signaling is essential for the nutrient sensing of the autophagy pathway (Mizushima *et al.*, 2004; Russell, Yuan and Guan, 2014).

Fluorescence microscopic analyses revealed that differential autophagic induction, following starvation, exists in different tissues. Although organ dependent, autophagy is induced in the majority of organs (including the heart) in response to nutrient starvation (Mizushima *et al.*, 2004; Moulis and Vindis, 2017).

The appropriate fasting time should ideally be determined empirically, but for nutrient starvation experiments rats are usually deprived of food for 24 hours, still allowing free access to water (Mizushima *et al.*, 2004; Mizushima, Yoshimori and Levine, 2010; Klionsky *et al.*, 2016).

Of importance is the question whether a shorter period of starvation also induces autophagy significantly, and thereby influences the results obtained following pharmacological manipulation of autophagy. The rats in our study had free access to food and water and it is unlikely that they would starve themselves for a period of 24 hours. It is, however, possible that we did experiments on rats that were “partially” starved. Rats are nocturnal animals,

preferring to eat at night (12hour dark cycle) and using rats for experimental purposes at the end of their “day cycle” may imply working with a short-term starvation object. Bringing the rat into the experimental laboratory might even prolong the “day cycle” and therefore the fasting period.

Whether short term fasting will make a difference to the results obtained is not known, but that the rats were not standardized from a nutritional perspective is a possibility. Another study demonstrated that, in nutrient deprived mice, LC3B expression fluctuated excessively among individuals, implying that autophagic induction was not synchronised (Pietrocola *et al.*, 2017). In an attempt to coordinate autophagic induction in all mice of an experimental group, Ezaki and colleagues successfully conducted a starvation/ feeding/ re-starvation regimen where mice were fasted for 24 h, then fed for 2 h in the dark to suppress autophagy to a minimal level and re-fasted to induce autophagy (Ezaki *et al.*, 2011). Keeping the circadian rhythm in mind, replicate experiments should preferably be conducted at the same time of day (Moulis and Vindis, 2017).

We purposefully did not want the rats to be fasted, since we were aiming to replicate a realistic human ischemic/reperfusion model. The corollary to this would be that the experimental subject was not fasted (and that the “medical intervention” only take place *after* the ischemia event).

Autophagy can also be induced by other physiological stressors; exercise, infection and hypoxia (Murrow and Debnath, 2013). None of these was relevant in our study. The influence of psychological stressors (noise, unnatural day/night cycle, human handling of the animals) on autophagic activation is however still unknown and would be very difficult to standardise and to investigate.

7.7.3. Experimental model in combination with the degree and duration of ischemia and reperfusion

It is well-established (and also shown in our study) that the ischemic and reperfusion duration play a significant role in study outcome and may therefore be responsible for the inconsistency in results (in terms of autophagic activity and cardioprotection). As referred to before, rabbit hearts only demonstrated an upregulation in autophagy following 40 min of ischemia. In the same model autophagic flux was impaired after 60 min of global ischemia (Decker and Wildenthal, 1980). Autophagic induction appears sooner in rat (30 min) (Hamacher-Brady *et al.*, 2007) and mice (20 min) (Matsui *et al.*, 2007) hearts. Reperfusion

times also varied between 15 -120 min in studies investigating autophagy using the same experimental model and animal (Hamacher-Brady *et al.*, 2007; Matsui *et al.*, 2007; French, Taatjes and Sobel, 2010). The preceding variables motivated us to have different options for ischemia (15 and 20 min), early reperfusion (10 and 30 min) and late reperfusion (60, 90 and 120 min) in our control experiments.

The very consistent and convincing autophagic upregulation following ischemia and reperfusion in our control hearts however disappeared with the addition of 3MA (as expected) and with Rapamycin (unexpectedly). This may indicate that, theoretically, our model, including the ischemia/reperfusion sequence, should be able to confirm or reject our hypothesis. However, more attention needs to be applied to measuring of autophagy and the drugs/methods used to manipulate autophagy.

Longer ischemic and reperfusion periods (for example an in vivo model) will be insightful and may supply answers to our questions. Autophaghic steady state and flux manipulation, respectively, may be more apparent with longer periods of (and further spaced) ischemia and reperfusion. In contrast to the isolated perfused rat heart, an in vivo model will allow for much longer reperfusion periods and may be ideal from that perspective.

7.8. SUMMARY

Our study confirmed that the isolated perfused rat heart can be used to study autophagy.

We were able to demonstrate that ischemia followed by reperfusion is responsible for an *increase* in autophagic steady state and flux. This increase was more pronounced:

- Following 20 min ischemia compared to 15 min ischemia.
- In late reperfusion as opposed to early reperfusion.

Increasing the reperfusion duration more may either further enhance autophagic induction or result in dysfunctional autophagy (an increase in steady state without an increase in flux). What we do know is that:

- In this model, autophagic flux was still intact following 20 min ischemia and 120 min reperfusion.
- A much longer reperfusion (or ischemic) duration cannot be successfully implemented in this model. (We unsuccessfully attempted 25 min of global ischemia.)

Autophagic manipulation did not result in any change in global myocardial function during reperfusion after ischemia. Insufficient autophagic changes in combination with the time constraints as a result of the model used were the most important contributing factors.

Autophagic inhibition (with 3MA) during early reperfusion resulted in regional myocardial protection, as demonstrated by the reduction in infarct size. Both Rapamycin dosages administered during early reperfusion also abolished the increase in autophagy demonstrated for this reperfusion period in the control experiments – this “decrease” in autophagic activity however did not result in a reduction in infarct size. A combination between conventional and alternative autophagic pathway inhibition, apoptotic increase and/or other drug related effects therefore may well be a reasonable explanation.

The demonstrated increase in autophagic flux following 250 nM Rapamycin in late reperfusion did not influence the infarct size.

We were unable to induce autophagy during early reperfusion. The reduction of autophagy during late reperfusion (3MA and 1 nM Rapamycin groups) was without effect on regional myocardial protection.

Having discussed the present study findings, the subsequent chapter (Chapter 8) draws conclusions and highlight the most important aspects and results based on the research. The limitations of this study and future directions of this research topic will also be considered.

CHAPTER 8
CONCLUSION

8.1. FINAL CONCLUSIONS

A successful treatment strategy against reperfusion injury is important since ischemic heart disease (and consequently reperfusion injury) are the main contributors to morbidity and mortality in the world (Mozaffarian *et al.*, 2015). Following our study, the "magic bullet" against reperfusion injury, administered *during reperfusion*, remains elusive. We aimed to replicate a realistic human model of ischemia and reperfusion, only administering the intervention (autophagic manipulation) during reperfusion.

The main findings in our study include:

a) The isolated perfused rat heart model can be successfully used to investigate autophagy.

b) Ischemia and reperfusion, in our model, are followed by a functional upregulation of autophagy. The increase in steady state *and* autophagic flux was more prominent following a longer ischemic (15 versus 20 min) and reperfusion (30 versus 120 min) duration.

c) The degree of autophagic manipulation in our study did not result in any significant change in global myocardial function. This might simply be because autophagic changes do not affect global myocardial function. Insufficient autophagic manipulation in combination with the time constraints of the model used are other relevant important contributing factors.

d) 3 Methyl-adenine administration during early reperfusion resulted in a reduction in infarct size. This can potentially be attributed to a combination of the reduction in the conventional and alternative autophagic pathways, an increase in apoptosis and other drug effects.

e) The induction of autophagy during late reperfusion using (250 nM Rapamycin) had no effect on cardioprotection. The autophagic induction in this group was however not very pronounced, and demonstrated only in the LC3 B/A ratio and p62 levels. It would therefore be unwise to draw a final and reliable conclusion in terms of the cardioprotective or –damaging effects of autophagic induction during late reperfusion.

We were unable to induce autophagy (with 1, 100 and 250 nM Rapamycin) during early reperfusion. The degree of autophagic inhibition during late reperfusion (in the 1 nM Rapamycin group) was very mild, and actually indicated that the upregulation seen in the control experiments was abolished. It is therefore not possible to draw conclusions (in terms of myocardial protection) regarding the effects of autophagic induction during early reperfusion, and autophagic inhibition during late reperfusion.

A novel finding was the fact that early reperfusion administered 3MA and 250 nM Rapamycin administered early as well as late, resulted in a decrease in flux of the alternative autophagic pathway (as suggested by Rab9).

The question remains, is the induction of autophagy during early reperfusion and the inhibition during late reperfusion cardioprotective? It is difficult to answer this question implied by our hypothesis. Following our results, we can report that:

- Autophagic inhibition during early reperfusion may have cardioprotective properties.
- The mild induction of autophagy during late reperfusion has no damaging effects to the hearts.

8.2. ROLE PLAYERS

The working rat heart model can be used to study the effect of autophagic manipulation. Although changes in autophagy were convincing in the control experiments, the results in the interventional experiments were less conclusive. The limitations associated with this model may have contributed to this. A longer reperfusion period might have given more time for the drug effects (especially Rapamycin) to become apparent. The changes in autophagy might have also been more pronounced if ischemia and reperfusion were even longer, facilitating manipulation. Our experimental model however did not allow us to prolong the ischemic time to more than 20 min and the reperfusion time to much more than 120 min respectively.

Another potential confounding factor in terms of our model is the lack of standardisation in terms of the nutritional status of the rats. This may be important since starvation is the most potent physiological autophagic inducer known. We however purposefully allowed the rats free access to food and water since we were aiming to replicate a realistic human ischemic/reperfusion model.

The difficulties in measurement of autophagy also contributed to the controversies in our

results. Perfect protein assays unfortunately do not exist.

Factors influencing the accuracy and the reproducibility of protein assays include the following: The rat itself as well as its living conditions, circumstances surrounding perfusion of the heart, lysate preparation, the multiple factors influencing accuracy of the specific protein (as discussed in Chapter 7), the antibody used and the various steps involved in western blotting. Lysates, for example, may undergo mechanical, temperature and oxidative stress, which all contribute to autophagic induction and therefore artefacts (Klionsky *et al.*, 2012).

The other autophagic measuring methods (electron microscopy and immunofluorescence) also have limitations, there is therefore no single "gold standard" to monitor autophagic activity (Mizushima, Yoshimori and Levine, 2010). It is advisable to employ several different autophagic proteins during western blotting in combination with other measuring methods to accurately and reliably assess autophagic activity.

Pharmacological manipulation of autophagy played the most problematic role in our study. Again, the perfect method to activate or inhibit the autophagy pathway does not exist (Mizushima, Yoshimori and Levine, 2010). We elected to use of the most commonly used autophagic inhibitor (3MA) and inducer (Rapamycin) (Seglen and Gordon, 1982; Klionsky *et al.*, 2016). The limitations of both (as discussed in Chapter 7, Section7.5), in combination with the disappointing results (especially with Rapamycin) prompt us to advise against using Rapamycin in similar future studies. Wortmannin as an autophagic inhibitor (to avoid the dual role of 3MA (Wu *et al.*, 2010)) and an ATP-competitive inhibitor like Torin1 as autophagic activator may be preferred. Torin1 is more potent and would be able to block Rapamycin resistant mTORC1 functions, as discussed in Chapter 7 (Thoreen and Sabatini, 2009). Making use of genetic manipulation to confirm the response to pharmacological induction and inhibition would be the gold standard.

Apoptosis and stress also play an influencing role. The degree of stress cells undergoes may determine if autophagy, apoptosis, necrosis or a combination of the three are going to take place. The cross-talk between apoptosis and autophagy, as discussed in Chapter 4 and 7, is quite complex. Measuring apoptotic activity in autophagy related studies is important for the following reasons:

- The activity level of the one will influence the other.

- Both of them play a critical role in cell death, and will therefore influence measured global and regional myocardial function.

8.3. LIMITATIONS AND STRENGTHS

The main limitations accompanying our study include the following:

- Rapamycin did not induce autophagy during early reperfusion.
- Although all the rats had free access to food and water, a standardised nutritional state cannot be guaranteed.
- Western blotting only was used to monitor autophagic activity.
- We did not monitor apoptotic activity in parallel with autophagic activity.
- Reperfusion duration limitation.

The strengths of our study include:

- We made use of a validated, commonly used model and experimental animal.
- We made use of different ischemic and reperfusion periods, in an attempt to achieve the most suitable model for autophagic manipulation.
- An array of autophagic proteins (6 in total) was used hoping to compensate for specific, and to avoid overlapping, limitations.
- We assessed autophagic steady state *and* flux.
- Our intervention that was administered during reperfusion only, and at two different stages of reperfusion is novel and relevant from a clinical point of view.

8.4. FUTURE DIRECTIVES

Standardisation of stress and nutritional states of the experimental animal and more than one autophagic measurement technique will contribute to more reliable and reproducible similar studies in the future. Parallel monitored apoptotic activity will enable differentiating between the contribution of autophagy and apoptosis on myocardial protection respectively. An in vivo model allowing for a longer reperfusion period and time between intervention and (autophagic and infarct size) measurement would also be strongly advised. It would give the drug/intervention the opportunity to reach a maximum effect and would therefore be a more accurate reflection of its effect on autophagy (and therefore potential cardioprotection).

In terms of autophagy manipulation; different dose/response, administration route and

exposure duration studies have to be investigated. More than one method to induce or inhibit autophagy will enable differentiation between pleiotropic drug effects or autophagic manipulation as the cause for observed results. In view of Rapamycin's relatively weak autophagic inducing effect and the time dependency associated with its use, we would advise against the use of Rapamycin as autophagic inducer in our experimental model. Lastly, genetic confirmation of drug manipulation would be ideal.

Appendix

Appendix A: Control experiments: Effect of ischemic (15 min vs 20 min global ischemia) and reperfusion time (30 min vs 70 min) on functional recovery

	15min GI Control			20min GI Control		
	Pre-ischemia	**30'R**	**70'R**	**Pre-ischemia**	**30'R**	**70'R**
Qc (ml/min)	14.64 ±0.64	13.73±0.89	15.75±1.44	12.85±0.65	11.15±0.47	9.95±0.57
Qa (ml/min)	43.42±1.66	27.6±2.27	19.4±1.52	38.4±1.33	9.82±1.8	4.28±1.75
CO (ml/min)	58.15±1.96	41.4±2.08	35.1±1.66	51.25±1.28	20.67±1.86	14.23±2.19
PSP (mmHg)	95.7±0.9	92±1,16	89.1±0.46	92.2±0.63	86.9±1.02	83.9±1.57
HR (beats/min)	283±10.23	294.9±12.33	303.4±6.06	299.9±10.33	265±10.09	271.6±10.1
Wtot (mWatts)	12.38±0.44	8.44±0.51	6.96±0.33	10.5±0.33	4.02±0.4	2.69±0.45

Working heart data following 15 min stabilisation (the pre-ischemic period), 30 min and 70 min reperfusion. Data presented as mean ± SEM. (n=5-10) Abbreviations: HR: heart rate, Qa: aortic outflow, Qe: coronary flow rate, Wtot: work total, CO: cardiac output, PSP: peak systolic pressure, R: reperfusion, GI: global ischemia and Pre-I: pre-ischemic interval.

Appendix B: Control experiments: Effect of chloroquine pretreatment on functional recovery during reperfusion (30 and 70min) after 15 and 20min global ischemia

	15'GI Control with CQ			20'GI Control with CQ		
	Pre-ischemia	**30'R**	**70'R**	**Pre-ischemia**	**30'R**	**70'R**
Qc (ml/min)	13.85±0.75	10.45±1.07	11.15±1.46	15.75±0.42	10.45±1.35	9.70±1.21
Qa (ml/min)	47.80±1.99	28.70±4.06	24.80±4.94	40.40±1.36	1.80±0.70	0.92±0.49
CO (ml/min)	61.60±2.34	40.15±4.57	36.15±6.08	56.15±1.08	10.45±1.88	10.62±1.39
PSP (mmHg)	95.70±0.73	91.40±1.02	82.40±9.19	94.60±0.95	71.80±8.81	70.90±8.72
HR (beat/min)	257.60±8.15	225.4±17.86	232.6±26.24	290.2±9.90	247.6±35.05	247.6±31.28
Wtot (mWatts)	12.95±0.53	8.13±0.99	7.61±1.25	11.80±0.31	2.22±0.36	1.91±0.28

Working heart data following 15 min stabilisation (the pre-ischemic period), 30 min and 70 min reperfusion. Data presented as mean ± SEM. (n=5-10) Abbreviations: HR: heart rate, Qa: aortic outflow, Qe: coronary flow rate, Wtot: work total, CO: cardiac output, PSP: peak systolic pressure, R: reperfusion, GI: global ischemia and Pre-I: pre-ischemic interval.

Appendix C: Interventional experiments: Effect of 3MA, with and without Chloroquine pretreatment, on functional recovery during reperfusion following 20 min GI

	20'GI with 3MA			20'GI with 3MA and CQ		
	Pre-ischemia	**30'R**	**70'R**	**Pre-ischemia**	**30'R**	**70'R**
Qc (ml/min)	12.70±0.55	11.50±1.70	8.80±2.94	14.10±0.96	10.05±1.80	7.30±3.08
Qa (ml/min)	40.20±1.28	8.92±3.07	2.80±1.74	42.80±1.77	7.80±2.53	0.80±0.80
CO (ml/min)	52.90±1.52	20.42±4.25	11.54±4.59	56.90±1.87	17.63±3.74	10.10±2.67
PSP (mmHg)	96.60±1.56	78.30±8.81	65.00±16.48	96.90±1.49	67.80±11.43	55.20±14.78
HR (beat/min)	266.70±11.92	227.80±27.77	201.20±52.34	294.20±13.58	217.9±37.13	239.4±61.38

Wtot (mWatts)	11.38±0.47	4.03±0.90	2.17±0.91	12.27±0.54	3.48±0.83	1.53±0.42

Working heart data following 15 min stabilisation (the pre-ischemic period), 30 min and 70 min reperfusion. Data presented as mean ± SEM. (n=5-10) Abbreviations: HR: heart rate, Qa: aortic outflow, Qe: coronary flow rate, Wtot: work total, CO: cardiac output, PSP: peak systolic pressure, R: reperfusion, GI: global ischemia and Pre-I: pre-ischemic interval.

Appendix D: Interventional experiments: Effect of 1 nM Rapamycin, with and without Chloroquine pretreatment, on functional recovery during reperfusion following 20 min GI

	20'GI with Rapa			20'GI with Rapa and CQ		
	Pre-ischemia	**30'R**	**70'R**	**Pre-ischemia**	**30'R**	**70'R**
Qc (ml/min)	15.25±0.88	10.70±1.43	11.40±2.05	14.70±0.63	9.80±1.74	9.60±2.81
Qa (ml/min)	40.20±1.72	5.72±1.39	2.84±1.05	42.80±1.34	5.80±2.08	3.70±2.23
CO (ml/min)	55.45±1.96	16.42±2.24	14.24±2.73	57.50±1.65	15.60±3.17	13.30±3.76
PSP (mmHg)	97.20±1.74	75.40±8.45	77.00±3.39	98.20±0.90	66.20±11.30	63.4±16.03
HR (beat/min)	267.20±10.10	201.70±29.06	249.20±10.35	261.90±8.59	188.9±33.22	193.4±49.9
Wtot (mWatts)	12.00±0.57	2.70±0.52	2.51±0.54	12.54±0.41	2.73±0.68	2.37±0.70

Working heart data following 15 min stabilisation (the pre-ischemic period), 30 min and 70 min reperfusion. Data presented as mean ± SEM. (n=5-10) Abbreviations: HR: heart rate, Qa: aortic outflow, Qe: coronary flow rate, Wtot: work total, CO: cardiac output, PSP: peak systolic pressure, R: reperfusion, GI: global ischemia and Pre-I: pre-ischemic interval.

Appendix E: Interventional experiments: Effect of 250 nM Rapamycin, with and without Chloroquine pretreatment, on functional recovery during reperfusion following 20 min GI

	20'NIKA with 250 nM Rapa			20'NIKA with 250 nM Rapa and CQ		
	Pre-ischemia	**30'R**	**80'R**	**Pre-ischemia**	**30'R**	**80'R**
Qc (ml/min)	15.00±0.30	11.87±0.60	9.75±0.97	13.40±0.72	11.10±0.40	9.80±0.58
Qa (ml/min)	40.22±1.68	14.50±3.69	8.20±3.22	39.00±1.56	16.80±3.01	5.60±2.32
CO (ml/min)	55.22±1.89	26.38±3.21	18.00±3.16	52.40±2.22	27.90±2.69	15.40±2.42

PSP (mmHg)	97.44±1.86	87.50±2.59	84.25±1.44	96.50±2.11	84.20±1.36	77.60±2.40
HR (beat/min)	289.66±10.08	266.50±19.33	262±14.66	251.70±12.07	236.60±7.29	246.20±7.39
Wtot (mWatts)	11.98±0.55	5.18±0.79	3.40±0.65	11.25±0.57	5.25±0.59	2.70±0.48

Working heart data following 15 min stabilisation (the pre-ischemic period), 30 min and 70 min reperfusion. Data presented as mean ± SEM. (n=5-10) Abbreviations: HR: heart rate, Qa: aortic outflow, Qe: coronary flow rate, Wtot: work total, CO: cardiac output, PSP: peak systolic pressure, R: reperfusion, GI: global ischemia and Pre-I: pre-ischemic interval.

Appendix F: Isolated working rat heart data for control groups (following 15 min and 20 min ischemia), as well as the interventional experiments following 3 Methyl-adenine, 1 nM Rapamycin and 250 nM Rapamycin administration

This includes heart rate (HR in beats per minute), coronary flow rate (Qc, in mL / min), aortic output (Qa, in mL / min), total cardiac output, (CO in mL / min), peak systolic pressure, (PSP in mmHg) and total work (Wtot in mWatts) recorded following global ischemia. All data are presented as mean ± SEM. Abbreviations: HR: heart rate, Qa: aortic outflow, Qe: coronary flow rate, Wtot: work total, CO: cardiac output, PSP: peak systolic pressure, R: reperfusion, GI: global ischemia and Pre-I: pre-ischemic interval.

Isolated working rat heart perfusion data for **15 min global ischemia control experiments** (with and without Chloroquine) for early and late reperfusion.

15'NIKA Control group	Heart number	PI Qc	30'R Qc	70'R Qc	Qa	30'R Qa	70'R Qa	CO	30'R CO	70'R CO	PSP	30'R PSP	70'R PSP	HR	30'R HR	70'R HR	Wtot	30'R Wtot	70'R Wtot
	1	13.5	19.5	25	40	28	16	53.5	47.5	41	93	92	89	280	256	291	10.946	9.7	8.13
	2	15	12	12	48	36	28	63	48	40	93	92	89	288	291	300	13.006	9.7	7.9
	3	15	15	11	46	20	20	61	35	31	94	90	91	319	357	265	12.729	7.03	6.26
	4	16.5	11.3	12	48	30	16	64.5	41	28	98	92	91	283	278	312	14.032	8.37	5.66
	5	16.5	11	12	48	36	16	64.5	47	28	98	92	89	303	232	323	14.032	9.6	5.53
	6	16.5	14	16	34	34	24	50	48	40	101	101	89	242	309	309	11.34	10.76	7.9
	7	11	12	16.5	36	32	24	47	44	40	93	94	90	259	261	297	9.72	9.18	8.14
	8	16.5	16.5	18	42	22	20	60.5	38.5	38	94	90	89	337	312	288	12.68	7.7	7.51
	9	12	15	21	44	16	12	56	31	33	95	87	86	294	341	326	11.92	5.8	6.3
	10	13.5	11	14	48	22	18	61.5	33	32	98	90	88	234	312	323	13.38	6.6	6.25
	Average	14.6	13.73	15.75	43.4	27.6	19.4	58.15	41.3	35.1	95.7	92	89.1	283.9	294.9	303.4	12.3785	8.444	6.958
	SE	0.64	0.89	1.44	1.66	2.27	1.52	1.96	2.08	1.66	0.9	1.16	0.46	10.23	12.33	6.06	0.44	0.51	0.33

15'GI Control group	Heart number	PI Qc	30'R Qc	70'R Qc	Qa	30'R Qa	70'R Qa	CO	30'R CO	70'R CO	PSP	30'R PSP	70'R PSP	HR	30'R HR	70'R HR	Wtot	30'R Wtot	70'R Wtot
with CQ	153	12	9	9	36	10	4	48	19	13	91	86	88	288	213	227	9.696	3.627	2.539
	152	12	10.5	9	52	38	34	64	48.5	43	97	91	92	233	238	246	13.781	9.797	8.782
	151	13.5	10.5	12	56	40	42	69.5	50.5	54	98	91	93	297	248	263	15.12	10.2	11.148
	150	13.5	3	15	52	44	42	65.5	57	57	97	95	93	233	234	268	14.104	12.021	11.768
	149	13.5	16	16.5	48	42	38	61.5	58	54.5	94	93	92	256	250	254	12.833	11.974	11.131
	148	13.5	10.5	14	52	20	24	65.5	30.5	40	96	89	90	278	268	259	13.959	6.026	7.992
	147	15	12	10.5	46	30	18	61	42	28.5	98	92	88	263	73	280	13.271	8.578	8.578
	146	11	9	0	40	15	0	51	24	0	93	88	0	224	222	0	10.529	4.688	0
	145	19.5	10.5	12	52	14	12	71	24.5	24	97	97	97	270	270	270	13.67	4.717	4.6
	144	15	13.5	13.5	44	34	34	59	47.5	47.5	96	92	91	234	238	259	12.574	9.701	9.6
	Average	13.85	10.45	11.15	47.8	28.7	24.8	61.6	40.15	36.15	95.7	91.4	82.4	257.6	225.4	232.6	12.95	8.13	7.6138
	SE	0.75	1.07	1.46	1.99	4.06	4.94	2.34	4.57	6.08	0.73	1.02	9.19	8.15	17.86	26.24	0.53	0.99	1.253

Isolated working rat heart perfusion data for **20 min global ischemia control experiments** (with and without Chloroquine) for early and late reperfusion.

20'GI Control group	Heart number	PI Qc	30'R Qc	70'R Qc	PI Qa	30'R Qa	70'R Qa	PI CO	30'R CO	70'R CO	PI PSP	30'R PSP	70'R PSP	PI HR	30'R HR	70'R HR	PI Wtot	30'R Wtot	70'R Wtot
	1	11	9	8	38	4	0	49	13	8	92	85	81	280	252	312	10.01	2.453	1.44
	2	15	10	14	46	2	14	61	12	28	96	80	88	286	180	248	13	2.13	5.47
	3	12	10.5	10	40	3.2	2	52	13.7	12	91	84	88	349	280	248	10.51	2.55	2.34
	4	10.5	11	8	44	14	0	54.5	23	8	95	89	82	278	288	312	11.49	4.54	1.46
	5	15	13	11	32	9	0	47	21	11	90	86	82	286	275	286	9.39	4	2
	6	16.5	14	9	34	6	0	50.5	20	9	91	88	73	288	270	283	10.2	3.91	1.46
	7	13.5	12	9	38	14	8.8	51.5	26	17.8	92	88	87	316	286	227	10.52	5.08	3.38
	8	12	10.5	9	38	14	0	50	24.5	9	90	89	82	360	265	254	9.99	4.84	1.64
	9	12	10.5	11	36	18	12	48	28.5	23	92	91	90	300	268	240	9.8	5.76	4.6
	10	11	11	10.5	38	14	6	49	25	16.5	93	89	86	256	286	306	10.12	4.94	3.15
	Average	12.85	11.15	9.95	38.4	9.82	4.28	51.25	20.67	14.23	92.2	86.9	83.9	299.9	265	271.6	10.5	4.02	2.69
	Standard error	0.65	0.47	0.57	1.33	1.8	1.75	1.28	1.86	2.19	0.63	1.02	1.57	10.33	10.09	10.11	0.33	0.4	0.45

20'GI Control group	Heart number	PI Qc	30'R Qc	70'R Qc	Qa	30'R Qa	70'R Qa	CO	30'R CO	70'R CO	PSP	30'R PSP	70'R PSP	HR	30'R HR	70'R HR	Wtot	30'R Wtot	70'R Wtot
with CQ	124	16.5	14	13	38	4	0	54.5	18	13	97	87	84	283	248	248	11.74	3.48	2.42
	123	14	7.5	8	48	0	0	62	7.5	8	96	70	79	240	180	234	13.21	1.17	1.4
	122	16.5	13	12	40	6	4	56.5	1	16	97	89	87	330	268	278	12.17	3.75	3.09
	121	15	11	10	40	2	0	55	13	10	93	89	83	330	330	312	11.36	2.57	1.84
	120	16.5	13	11	44	2	2	60.5	15	13	99	89	88	270	303	275	13.3	2.96	2.54
	119	13.5	9	9	46	4	3.2	59.5	13	12.2	93	86	82	263	222	224	12.28	2.48	2.22
	118	16	14	13	36	0	0	52	14	13	93	86	86	316	312	300	10.74	2.67	2.48
	117	16.5	13	12	36	0	0	52.5	13	12	91	67	72	319	316	303	10.606	1.933	1.918
	116	18	10	9	36	0	0	54	10	9	90	55	48	278	297	297	10.789	1.22	1.22
	115	15	0	0	40	0	0	55	0	0	97	0	0	273	0	0	11.84	0	0
	Average	15.75	10.45	9.7	40.4	1.8	0.92	56.15	10.45	10.62	94.6	71.8	70.9	290.2	247.6	247.6	11.8	2.22	1.91
	SE	0.43	1.35	1.21	1.36	0.7	0.49	1.08	1.88	1.39	0.95	8.81	8.72	9.9	35.05	31.28	0.31	0.36	0.28

Isolated working rat heart perfusion data following **3 Methyl-adenine** (with and without Chloroquine) administration during early and late reperfusion, after 20 min of global ischemia:

20'GI 3MA	Heart number	PI Qc	30'R Qc	70'R Qc	Qa	30'R Qa	70'R Qa	CO	30'R CO	70'R CO	PSP	30'R PSP	70'R PSP	HR	30'R HR	70'R HR	Wtot	30'R Wtot	70'R Wtot
Late reperfusion	183	12	7.5	7.5	44	6	0	56	13.5	7.2	100	80	78	222	185	211	12.432	2.397	1.246
	184	13.5	0	0	42	0	0	55.5	0	0	95	0	0	297	0	0	11.704	0	0
	185	10.5	8	6	32	4	0	42.5	12	6	89	83	73	242	240	229	8.397	2.211	0.972
	186	11	16	14	40	18	6	51	34	20	98	93	85	216	244	278	11.095	7.019	3.774
	187	16.5	16.5	16.5	42	30	8	58.5	46.5	24.5	98	94	89	297	280	288	12.727	9.703	4.84
Early reperfusion	203	13.5	11		36	0		49.5	11		89	84		341	323		9.78	2.051	
	204	12	12		42	3.2		54	15.2		103	87		259	242		12.347	2.935	
	205	12	18		38	10		50	28		95	86		259	280		10.545	5.345	
	206	12	11		46	16		58	27		103	91		259	250		13.262	5.454	
	207	14	15		40	2		54	17		96	85		275	234		11.508	3.207	
Average	**195**	12.7	11.5	8.8	40.2	8.92	2.8	52.9	20.42	11.54	96.6	78.3	65	266.7	227.8	201.2	11.38	4.03	2.17
SE	**3.37**	0.5487	1.703	2.94	1.28	3.074	1.743	1.5198	4.254	4.594	1.56	8.814	16.48	11.92	27.77	52.34	0.47	0.9	0.913

20'GI + 3MA/CQ	Heart number	PI Qc	30'R Qc	70'R Qc	PI Qa	30'R Qa	70'R Qa	PI CO	30'R CO	70'R CO	PI PSP	30'R PSP	70'R PSP	PI HR	30'R HR	70'R HR	PI Wtot	30'R Wtot	70'R Wtot
Late reperfusion	188	18	0	0	40	0	0	58	0	0	93	0	0	341	0	0	11.974	0	0
	189	12	11	0	48	12	4	60	23	14	102	85	81	300	280	273	13.586	4.34	2.517
	190	16.5	11	11	48	2	0	64.5	13	11	97	84	78	366	256	326	13.889	2.453	1.904
	191	18	15	10.5	36	8.8	0	54	23.8	10.5	90	84	66	330	333	333	10.789	4.438	1.538
	192	16.5	16	15	36	0	0	52.5	16	15	91	71	51	306	270	265	10.606	2.521	1.698
Early reperfusion	208	15	15		52	20		67	35		104	90		254	248		15.468	6.993	
	209	12	10.5		46	2		58	12.5		100	84		291	278		12.876		
	210	10	0		38	0		48	0		94	0		250	0		10.016	0	
	211	12	12		44	20		56	32		99	90		270	268		12.307	6.393	
	212	11	10		40	11		51	21		99	90		234	246		11.208	4.195	
Average		14.1	10.05	7.3	42.8	7.58	0.8	56.9	17.63	10.1	96.9	67.8	55.2	294.2	217.9	239.4	12.27	3.48	1.53
SE		0.96	1.8	3.08	1.77	2.53	0.8	1.87	3.74	2.67	1.49	11.43	14.78	13.58	37.13	61.38	0.54	0.83	0.42

Isolated working rat heart perfusion data following **1nM Rapamycin** (with and without Chloroquine) administration during early and late reperfusion, after 20 min of global ischemia:

20'GI + Rapa	Heart number	PI Qc	30'R Qc	70'R Qc	PI Qa	30'R Qa	70'R Qa	PI CO	30'R CO	70'R CO	PI PSP	30'R PSP	70'R PSP	PI HR	30'R HR	70'R HR	PI Wtot	30'R Wtot	70'R Wtot
Late reperfusion	174	18	12	13.5	38	10	6	56	22	19.5	93	86	83	286	234	252	11.561	4.2	3.593
	175	18	18	18	40	6	3	58	24	21	94	86	81	278	68	229	12.103	4.582	3.776
	176	13.5	9	9	36	10	4	49.5	19	13	92	82	80	252	203	226	10.109	3.458	2.308
	177	15	10.5	10.5	40	2	1.2	55	12.5	11.7	108	77	77	194	250	256	13.186	2.136	1.999
	178(a)	14	0	6	36	0	0	50	0	6	92	0	64	250	0	283	10.212	0	0.852
Early reperfusion	193	19.5	13		36	2		55.5	15		98	80		300	278		12.074	2.93	
	194	14	10.5		48	10		62	20.5		98	88		297	238		13.488	0.004	
	195	10	10.5		34	12		44	22.5		93	85		290	256		9.084	4.245	
	196	14	10.5		50	2		64	12.5		104	83		250	256		14.776	2.303	
	197	16.5	13		44	3.2		60.5	16.2		100	87		275	234		13.431	3.128	
Average		15.25	10.7	11.4	40.2	5.72	2.84	55.45	16.42	14.24	97.2	75.4	77	267.2	201.7	249.2	12	2.7	2.51
SE		0.88	1.43	2.05	1.72	1.39	1.05	1.96	2.24	2.73	1.74	8.45	3.39	10.1	29.06	10.35	0.57	0.52	0.54

20'GI + Rapa/CQ	Heart number	PI Qc	30'R Qc	70'R Qc	PI Qa	30'R Qa	70'R Qa	PI CO	30'R CO	70'R CO	PI PSP	30'R PSP	70'R PSP	PI HR	30'R HR	70'R HR	PI Wtot	30'R Wtot	70'R Wtot
Late reperfusion	178(b)	16.5	16.5	16.5	42	10	4.5	58.5	26.5	21	95	84	80	283	268	270	12.337	4.941	3.729
	179	15	11	7.5	36	14	12	51	25	19.5	100	90	86	213	200	200	11.322	4.995	3.722
	180	16.5	13	11	46	0	0	62.5	13	11	97	80	71	265	224	234	13.458	2.308	1.733
	181	13	0	0	38	0	0	51	0	0	94	0	0	255	0	0	10.642	0	0
	182	16.5	10.5	13	42	6	2	58.5	16.5	15	97	80	80	273	254	263	12.597	2.93	2.664
Early reperfusion	198	14	11		40	0		54	11		96	81		268	234		11.508	1.978	
	199	15	12		46	14		61	26		103	93		240	199		13.948	5.367	
	200	10.5	10		42	14		52.5	24		101	89		233	204		11.771		
	201	13.5	0		46	0		59.5	0		99	0		286	0		13.076	0	
	202	16.5	14		50	0		66.5	14		100	65		303	306		14.763	2.02	
Average		14.7	9.8	9.6	42.8	5.8	3.7	57.5	15.6	13.3	98.2	66.2	63.4	261.9	188.9	193.4	12.54	2.73	2.37
SE		0.63	1.74	2.81	1.34	2.08	2.23	1.65	3.17	3.76	0.9	11.3	16.03	8.59	33.22	49.9	0.41	0.68	0.7

Isolated working rat heart perfusion data for control group as well as following **250nM Rapamycin** (with and without Chloroquine) administration during early and late reperfusion, after 20 min of global ischemia:

20'GI Control	Heart number	PI Qc	30'R Qc	80'R Qc	PI Qa	30'R Qa	80'R Qa	PI CO	30'R CO	80'R CO	PI PSP	30'R PSP	80'R PSP	PI HR	30'R HR	80'R HR	PI Wtot	30'R Wtot	80'R Wtot
Late reperfusion	335	16	14	11	34	4	0	50	18	11	101	90	88	248	233	246	11.21	3.59	2.15
	336	12	11	7.5	30	2	0	42	13	7.5	94	84	79	265	242	242	8.76	2.42	1.32
	337	13.5	12	10	38	20	10	51.5	32	20	101	93	88	254	246	242	11.55	6.61	3.91
	338	18	18	18	36	14	4	54	32	22	103	97	87	236	195	217	12.35	6.89	4.25
Early reperfusion	331	13.5			40			53.5			99			319			11.76		
	332	13.5			44			57.5			110			248			14.041		
	333	15			42			57			102			280			12.91		
	334	16			52			68			98			353			14.79		
Average		14.69	13.75	11.63	39.5	10	3.5	54.19	23.75	15.13	101	91	85.5	275.38	229	236.75	12.17	4.88	2.91
SEM		0.68	1.55	2.25	2.38	4.24	2.36	2.61	4.87	3.49	1.63	2.74	2.18	14.37	11.65	6.65	0.66	1.11	0.7

20'GI + Rapa	Heart number	PI Qc	30'R Qc	80'R Qc	PI Qa	30'R Qa	80'R Qa	PI CO	30'R CO	80'R CO	PI PSP	30'R PSP	80'R PSP	PI HR	30'R HR	80'R HR	PI Wtot	30'R Wtc	80'R Wtot
Late reperfusion	345	15	11	9	46	24	16	61	35	25	106	95	88	238	211	227	14.35	7.38	4.88
	346	15	11	7.5	44	14	4	59	25	11.5	97	86	82	288	270	254	12.7	4.77	2.09
	347	16.5	13.5	12	44	6	2	60.5	19.5	14	95	83	82	306	297	270	12.759	3.59	2.55
	348	15	12	10.5	38	14	11	53	26	21.5	92	86	85	306	288	297	10.82	4.96	4.06
Early reperfusion	339	14			40			54			100			306			11.99		
	340	16			46			62			100			309			13.76		
	341	15			36			51			104			238			11.77		
	342	13.5			32			45.5			89			316			8.99		
	343	15			36			51			94			300			10.64		
Average	343.33	15	11.87	9.75	40.22	14.5	8.2	55.22	26.38	18	97.44	87.5	84.25	289.66	266.5	262	11.98	5.18	3.4
SEM	1.07	0.3	0.6	0.97	1.68	3.69	3.22	1.89	3.21	3.16	1.86	2.59	1.44	10.08	19.33	14.66	0.55	0.79	0.65

20'GI + Rapa/CQ	Heart number	PI Qc	30'R Qc	80'R Qc	PI Qa	30'R Qa	80'R Qa	PI CO	30'R CO	80'R CO	PI PSP	30'R PSP	80'R PSP	PI HR	30'R HR	80'R HR	PI Wtot	30'R Wtc	80'R Wtot
Late reperfusion	354	13.5	10	9	38	22	10	51.5	32	19	94	87	83	254	216	233	10.75	6.18	3.5
	355	16.5	10.5	10	46	26	12	62.5	36.5	22	101	88	81	250	227	256	14.01	7.13	3.96
	356	15	12	12	40	12	4	55	24	16	100	82	78	254	252	259	12.21	4.37	2.77
	357	11	12	9	34	12	2	45	24	11	89	82	77	259	254	259	8.89	4.37	1.88
	358	12	11	9	34	12	0	46	23	9	90	82	69	246	234	224	9.19	4.19	1.38
Early reperfusion	349	14			44			58			95			265			12.23		
	350	10.5			36			46.5			112			180			11.56		
	351	15			44			59			97			323			12.71		
	352	16			42			58			95			278			12.23		
	353	10.5			32			42.5			92			208			8.68		
Average		13.4	11.1	9.8	39	16.8	5.6	52.4	27.9	15.4	96.5	84.2	77.6	251.7	236.6	246.2	11.25	5.25	2.7
SEM		0.72	0.4	0.58	1.56	3.01	2.32	2.22	2.69	2.42	2.11	1.36	2.4	12.07	7.29	7.39	0.57	0.59	0.48

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